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Antimicrobial Resistance of Staphylococcus aureus in the Northern West of Portugal*:*

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3- **Rachid Hnini**, Said Ihbour, Lhou Ouhida, Mohammed Chigr, Mohamed Merzouki, Lahoucine Bahi, Ahmed Gammouh, Mohamed Najimi and Fatiha Chigr. The physicochemical quality of raw milk sold at dairies of Beni Mellal city (Morocco). International Journal of Scientific & Engineering Research Volume 9, Issue 12, December-2018 ISSN 2229-5518.

4- **Rachid Hnini**, Said Ihbour, Lhou Ouhida, Hasna Merzouki, Ahmed Ait Chaoui, Fatiha Chigr and Mohamed Najimi. Evaluation of the Microbiological Quality of Different Moroccan Drinking Water in the Beni Mellal Region. Journal of Geoscience and Environment Protection, 7, 105-115. https://doi.org/10.4236/gep.2019.75011.

5- Nadia Ferdous, **Rachid Hnini,** Mouhamed Merzoukiki, Lhoucine Bahi, FatihaChigr, Mohamed Najimi. Microbiological Characteristics of Raw Cow Milk in Beni Mellal Area (Morocco). International Journal of Science and Engineering Applications Volume 6 Issue 10, 2017, ISSN-2319-7560.

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7- S. Ihbour, **R. Hnini**, M. El Habi, F. Chigr, M. Najimi. Dyslexia in Morocco: An overview of recent research. IOSR Journal of Research & Method in Education (IOSR-JRME) e- ISSN: 2320–7388,p-ISSN: 2320–737X Volume 9, Issue 1 Ver. I. (Jan. – Feb. 2019), PP 24-26.

8- Said Ihboour, **Rachid Hnini**, Hammou Anarghou, Ahmed Tohami Ahami, Fatiha Chigr, Mohamed Najimi**.** Diagnostic Of Dyslexic Disorders And Identification Of Factors Associated With Reading Learning Disabilities Within The Moroccan Context. ACTA NEUROPSYCHOLOGICA. Vol 17, No 3, 2019, 261-281.

9- Lhou Ouhida, **Rachid Hnini**, Said Ihbour, Fatiha Chigr, Mohamed Najimi. Physicochemical Quality of Cow Raw Milk Produced in the Fkih Ben Saleh Area. International Journal of Advances in Scientific Research and Engineering (ijasre). Volume 5, Issue 6 June – 2019.

10- Ismail Karaoui, Abdelkrim Arioua, Abdelkhalek El Amrani Idrissi, Wafae Nouaim, Driss Elhamdouni, Kamal Ait Ouhamchich, Mohammed Hssaisoune, **Rachid Hnini**. Evaluation of the River-Shallow Aquifer Exchange Process Effect on Surface Water Quality Deterioration —Case Study of Oued El Abid River Downstream Course, Morocco. Journal of Geoscience and Environment Protection, 2017, 5, 123-134.

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16'- Merzouki Hasna, Hnini Rachid, Brhich Amina, Ellaite Mohammed, Hanine Hafida, Lekhlif Brahim, Mandi Laila, Bahi Lahoucine, Merzouki Mohammed**.** Study of coagulation process with Lime in treat-ment of landfill leachate from Fkih Ben Salah city (Morocco). **Submitted to publication**

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Participation in National and International Congress

Communications

- Assessment of antimicrobial activities of Origanum elongatum and Cupresus atlantica extracts. **Rachid HNINI**, Souad MOUJAHID, Hanane OUALILI, Fatima CHIBI, Ibtissam SABIR, Chafik El Ouariaghli, Mariama LASKI, Rachid NMILA, Halima RCHID, Fatiha CHIGR and Mouhamed NAJIMI. **Communication Oral in** 7ème Symposium International sur les Plantes Aromatiques et Médicinales **SIPAM 2019**. **Faculté des sciences d'El jadida.**

- Beta-lactam antimicrobials activity and the diversity of *blaZ* gene in *Staphylococcus aureus* isolates from bovine mastitis in the northwest of Portugal. **Rachid Hnini**, Eliane Silva, Mouhamed Najimi and Gertrude Thompson. **Communication Oral in** 2ème congrès International des Sciences Pharmaceutiques 17-18 Février 2017. **FST de Béni mellal.**

- Effect of stress on animal production. **R. Hnini**, L. Ouhida, M. Bensaleh, M. Merzouki. **Communication** in International symposium on stress, addiction, and obesity. **Faculty of Medicine of Marrakech, Morroco December 3/5/2015.**

- Bovine *S. aureus* antimicrobial susceptibility and *blaZ* gene diversity. **Rachid Hnini**, Eliane Silva, Mouhamed Najimi and Gertrude Thompson. **Communication (Poster)** 1ère conférence internationale en Biotechnologie ? **Faculté Polydisciplinaire de Béni mellal**.

- Effet du stress chronique et systeme immunitaire chez la souris. **Hnini R.**, Chaoui N., Chigr F., Najimi M. **Communication (Poster)** in 1er symposium international des jeunes chercheurs en Neurosciences. **Faculté Polydisciplinaire de Béni mellal**.

- Evaluation de la valeur nutritionnelle du lait cru des vaches locales et améliorées. **Rachid HNINI**, Fatiha CHIGR, Mouhamed NAJII. **Communication (Poster)** 1ère édition des journées doctoriales organisée à la FST de Béni mellal sous le thème Recherche Scientifique : Innovation et Développement.

- Exploration de la diversité microbienne des rivières Marocaines par l'approche métagénomique. ETTAMIMI Sara, HAMMANI Khalil, GHAZAL Hassan, CHIGR Fatiha, NAJIMI Mouhamed, **HNINI Rachid** and ESSAYEH Soumya. Colloque International sur Applications des technologies Géospatiales en Géosciences. **Faculté Polydisciplinaire de Taza.**

Summary

In veterinary and human medicine, antimicrobial agents play an important role in the therapy of bacterial infections. The discovery of antimicrobial agents during the 20th century was considered one of humanity's most miraculous discoveries, but the gradual increase in antimicrobial resistance that has accompanied their use in hospitals, the community, animals and the environment has also been increased and has once again challenged the notion of the efficacy of antibiotics and their use in human medicine being or veterinary. In the first part of this study, we investigate the antimicrobial susceptibility of *Staphylococcus aureus* (*S. aureus*) isolates in the northwest of Portugal against antibiotics belonging to the β-lactam family; detect and study the *blaZ* and *mecA* resistance genes diversity in positive isolates. The antimicrobial susceptibility tests were performed by the disk diffusion method. The detection of *blaZ* and *mecA* was performed using specific PCR and the diversity of *blaZ* was evaluated by phylogenetic analysis. The antimicrobial susceptibility test showed different prevalence of phenotypic resistance and intermediate resistance by *S. aureus* to different antibiotics from β-lactam family such as penicillin, ampicillin, oxacillin, cefazolin and amoxicillin associate with clavulanic acid. However, piperacillin was the only antibiotics that have saved their effectiveness against all isolates of *S. aureus*. Concerning the results obtained for the *blaZ* and *mecA* resistance genes profiles. Among all tested *S. aureus* isolates, 67.3% were PCR positive for *blaZ* and negative for *mecA* genes, except for one isolate that was found to be positive for *mecA*. The *blaZ* gene phylogenetic analysis placed the studied *S. aureus* isolates selected for sequencing in 2 different clades, clade A and B, and they are closely related to different bovine mastitis and/or human *S. aureus* strains. Finally, *blaZ* phylogenetic analysis from *S. aureus* isolates showed diversity inside or between different herds in the northwest of Portugal. In the second part of this thesis, we have extended our study to evaluate *in vitro* the efficacy of a set of antimicrobials from other different families, such as Aminosides family, Tetracycline family, Macrolides family, Glycopeptides family and Lipopeptides family, more used in *S. aureus* infections treatment, to the resistance phenomenon and test related resistance genes among *S. aureus* isolates. The antimicrobial susceptibility tests were performed by the disk diffusion method. However, the detection of genes linked to the selected antimicrobials, such as *aph(3')-IIIa, ant(4`)-Ia and aac (6')-aph(2'')*, *tet(M)*, *tet(K)*, *erm(T)*, *van(A), lnu(C)*, *sal(A)*, *vga(C)* and *dfrK* were performed using specific PCR methods. The antimicrobial susceptibility testing demonstrated a different phenotypic resistance and intermediate resistance to Tetracycline, Erythromycin, Amikacine, Kanamycin, Clindamycin, Gentamycin, Tobramycin, Trimetroprim-sulfatomexazole and to Vancomycin was observed. Of the screened genes, *ant(4)-I-a* (63.5%), *tet(M)* (57.7%), *aph(3')-III-a* (30.8%), *dfrK* (19.2%), *tet(K)* (15.4%) and *lnu(C)* (5.8%) were detected. The remaining tested genes were not detected. The antimicrobials used in this study showed a high efficacy towards the *S. aureus* isolates collected during the period from 2003 to 2008. Whilst the isolates collected in 2017 were almost all phenotypic resistant or intermediate against all antimicrobials, they show an alarming and dramatic evolution of appearance of new resistant strains. In conclusion, these findings could be advantageous generally for design a new specific program for bovine mastitis disease control caused by *S. aureus* in the northwest region of Portugal.

Extended Summary

In veterinary and human medicine, antimicrobial agents play an important role in the therapy of bacterial infections. The discovery of antimicrobial agents during the 20th century was considered one of humanity's most miraculous discoveries, but the gradual increase in antimicrobial resistance that has accompanied their use in hospitals, the community, animals and the environment has also been increased and has once again challenged the notion of the efficacy of antibiotics and their use in human medicine being or veterinary. Over time, introductions of new classes of antimicrobial drugs have been manifested, often rapidly, by the emergence of resistant microorganisms. Worldwide, bovine mastitis is considered to be a common disease, touching dairy cows with high incidence. *Staphylococcus aureus* (*S. aureus*) is a Gram positive bacteria considered as an important human pathogen and known as one of the most important agents causing disease related with bovine mastitis in the world. Mastitis, important disease encountered in dairy herds, consists of an inflammation of the mammary gland, usually developed in response to intramammary bacterial infection. *S. aureus* is of particular importance, because it is highly infectious and it is characterized by significantly lower cure levels in comparison with infections caused by other microorganisms.

The main objective of this thesis is to approach mechanisms promoting resistance phenomena encountered in *S. aureus* bacteria by using a set of genotypic and phenotypic tests, cases of *S. aureus* isolated from mastitis in northwest of Portugal. Neverthless, the major objective aims to approach resistance phenomenon mechanisms by using genotyping and phenotyping tests, particularly for the case of mastitis caused by *S. aureus* in the northern west of Portugal. Thus; in the first part of this thesis, we studied the antimicrobial activity of beta-lactam antibiotics and the diversity of the *blaZ* gene in *S. aureus* isolates received from bovine mastitis of northwestern Portugal in order to show the mechanisms by which *S. aureus* can confer resistance to beta-lactam antibiotics despite the presence of a wide variety of different antibiotic groups within this family. In general, our first study aimed to investigate the antimicrobial susceptibility of 52 *S. aureus* mastitis isolates obtained in the years 2003-2004, 2007-2008 and 2017 in northwest of Portugal against antibiotics belonging to the Beta lactams family; and to predict the prevalence, detection and study of the diversity of *blaZ* and *mecA* resistance genes in positive isolates was also one of the main objectives of this study.

The results obtained of antibiotic susceptibility test, from the isolates collected during 2003-2004 and 2007- 2008 periods, indicated that the resistance to aztreonam (Monobactames group) was noted in all of isolates, whereas the resistance to penicillin (Penicillins G group) was found considerably higher arriving at 76.9% followed by resistance to ampicillin (Aminopenicillins group) with 73.0%. The resistance against to amoxicillin and to oxacillin was only 3.8% while

the resistance to amoxicillin combined to amoxicillin/clavulanic acid was null (all from Aminopenicillins group). In addition, all strains showed a significantly higher susceptibility 100.0% for other antibiotics analyzed in parallel such as cefazolin (Cephalosporines group); piperacillin (Ureidopenicillins group); imipenem (Carbapenems group) and ticarcillin (Carboxypenicillins group). However, all tested isolates were PCR positive for *blaZ* gene and only isolate 25 was positive for *mecA* gene. The phylogenetic analysis of the detected *blaZ* placed the isolates in 3 different clusters that are closely related to other different bovine mastitis and human *S. aureus* strains. However, the findings obtained from new strains collected in the year of 2017 showed a new prevalence for all tested antibiotics such as penicillin (27.0%), ampicillin (34.6%), oxacillin (65.4%), amoxicillin combined to clavulanic acid (34.6%) and cefazolin (42.3%). In addition, an intermediate resistant rate of 11.5%, 3.8%, 3.8%, 19.2% and 3.8% has been found for these already described antibiotics, respectively. Finally, piperacillin antibiotic has always shown a permanent susceptibility of 100.0% against all these new collected strains. The comparison of these new findings with those mainly found in 2003-2005 and 2007-2008 periods are widely different. As a result, we observed a noticeable reduction especially for penicillin (27.0% in 2017 instead of 76.9% in 2003 to 2008 period) and for ampicillin (34.6% in 2017 instead of 73.0% in 2003 to 2008 period).

In conclusion of this part of this thesis, we have observed, particularly in the case of isolates collected from 2003 to 2008, a high prevalence of resistance in *S. aureus* strains tested against penicillin and ampicillin while the remaining antibiotic groups tested, within the beta-lactam family, showed 100.0% activity against this pathogenic microbe. In parallel, a prevalence of 100.0% was observed for *blaZ*, a gene encoding Beta-lactam resistance. However, the phylogenetic analysis placed the isolates within 3 clusters closely related to different bovine mastitis and human *S. aureus* strains, with isolate 2 being the most divergent. Within clusters 1 and 3, most isolates are related with high prevalence of penicillin and ampicillin resistance. The isolates with a phenotypic susceptibility of 100.0% are included in cluster/subcluster 1 and cluster 2. However; in case of new collected strains, we have noted a decrease in prevalence of both antibiotics penicillin and ampicillin in 2017. This could be explained, for example, by veterinary practices because of the high prevalence of resistance of *S. aureus* to penicillin and ampicillin in the world, many veterinarians have moved in the direction of avoiding as possible or definitively the use and prescription of these two well-known antibiotics in the treatment of bacterial infections, particularly for mastitis caused by *S. aureus* in view of presence of a bad reputation attributable to the resistance phenomena that marked the Beta-lactam family worldwide. Thus, this avoiding of use of these antibiotics in therapeutic practices for a long

period could be lead to the inactivation of the genes responsible for this resistance (such as *blaZ*, *mecA*), then inactivation of the resistance acquisition by *S. aureus* to these two antibiotics well known in medicine. As well, we have also observed the appearance of intermediate resistant strains within the new collected isolates during 2017 on the one hand, and the appearance of isolates that showed a resistance to a number of antibiotics such as amoxicillin combined to clavulanic acid and cefazolin that were 100.0% more effective against isolates collected during years of 2003 to 2008 on the one hand. Finally, piperacillin antibiotic saved its effectiveness against older strains collected in 2003 to 2008, and it is also found to be very effective against new strains that have been recently collected in 2017. What needs to reconsider this antibiotic in order to fight and treat *S. aueus* infections originated from bovine mastitis? The new *blaZ* phylogenetic analysis from all *S. aureus* isolates collected in the years of 2003, 2004, 2007, 2008 and 2017 shown diversity inside or between different herds in the northwest of Portugal.

In the second part of this thesis, we have tried to extend our study to include and recover other antibiotics from other families in order to have an overview of the resistance profiles of *S. aureus* from bovine mastitis in northwest Portugal. Thus, the objective of this second part was to detect certain genes encoding *S. aureus* resistance on the one hand, and to predict the prevalence and evaluate in vitro the efficacy of a set of antibiotics against strains of *S. aureus* isolated from bovine mastitis in northwest Portugal on the other hand. For this reason, the antimicrobial susceptibility tests were also performed by the disk diffusion method while the detection of genes such as aminoglycoside-modifying enzymes (*aph(3')-III-a, ant(4)-I-a and aac(6')-aph(2')*), *tetM*, *tetK*, *ermT*, *vanA*, *spc*, *lnuC*, *salA*, *vgaC* and *dfrK*, was also performed using specific PCRs methods. The obtained findings from this study showed that of the 52 *S. aureus* isolates, 39 (75.0%), 35 (67.3%), 26 (50.0%), 22 (42.3%), 19 (36.5%), 17 (32.7%), 15 (28.8%), 12 (23.1%) and 11 (21.2%) showed high resistance in particular to tetracycline, erythromycin, vancomycin, amikacin, kanamycin, clindamycin, gentamycin, tobramycin and trimetroprim-sulfatomexazole, respectively. On the other hand, 18 (34.0%), 15 (28.8%), 14 (26.9%), 11 (21.2%), 7 (13.5% each), and 4 (7.7% each) showed intermediate resistance to vancomycin, trimetroprim-sulfatomexazole, tobramycin, gentamycin, tetracycline, erythromycin, kanamycin, clindamycin and amikacin, respectively. Of the screening genes, *ant(4)-I-a*, *tetM*, *aph(3')-II I-a*, *dfrK*, *tetK*, *lnuC* and *spc* were detected in 33 (63.5%), 30 (57.7%), 16 (30.8%), 10 (19.2%), 8 (15.4%), 3 (5.8%) and 1 (1.9%), respectively. Moreover, the remaining tested genes such as *vanA*, *ermT*, *salA*, *vgaC*, *aac (6') - aph (2')* were all found negatives. All of the antibiotics used in this study showed remarkable and a high efficacy

towards all the strains collected mainly during the period from 2003 to 2008. Whilst, all the new strains collected more recently in 2017 were, in most cases, resistant or intermediate against all these tested antibiotics, showing therefore an alarming and dramatic evolution of appearance of new very resistant strains leading to think immediately to definitively change our habitual vision and reaction to each use of these antibiotics before that humanity declares a total capitulation to new infections caused by *S. aureus* in the few coming years. In conclusion of this second investigation, our results mainly illustrated the presence of a high prevalence more than 50.0% phenotypic resistance in the tested *S. aureus* isolates against tetracycline, erythromycin and also for vancomycin. Moreover, a high prevalence of 63.5% and 57.7% was detected, by using PCR, for *ant(4)-I-a and tetM,* respectively. The findings presented from this study could be advantageous for designing specific programs of control for bovine mastitis disease caused by *S. aureus*. On the other hand; we have observed, in a certain isolates, that the findings obtained have confirmed that the proportion of *S. aureus* isolates with phenotypic resistance does not correspond to that of isolates identified with the genes detected, particularly in case of *blaZ*, *ermT*, *tet* and aminoglycosides-modifying enzymes genes because, for example, certain strains of *S. aureus* were found with 100.0% phenotypic sensitivity for all antibiotics tested also carry the *blaZ* gene. Similarly, results were almost observed for the rest genes such as *ermT*, tet and aminoglycosides-modifying-enzymes genes. The explanation of this phenomenon is more detailed in the discussion part.

Résumé

En médecine vétérinaire et humaine, les agents antimicrobiens jouent un rôle important dans le traitement des infections bactériennes. Leur découverte au cours du XXe siècle a été considérée comme un miracule en soi, mais l'augmentation progressive de la résistance aux antimicrobiens qui a accompagné leurs utilisations dans les hôpitaux, au sein de la communauté, des animaux et dans l'environnement a bouleversé de nouveau la notion de l'efficacité des antibiotiques et leurs utilisations en médicine humaine ou vétérinaire. Au fil du temps, l'introduction de nouvelles classes de médicaments antimicrobiens s'est manifestée, souvent et rapidement, par l'émergence de microorganismes résistants. Dans ce registre le cas de la mammite bovine considérée comme l'une des maladies courantes, touchant les vaches laitières à forte incidence en est une bonne illustration. Le *Staphylococcus aureus* (*S. aureus*) bactérie à Gram positif considérée comme un pathogène humain important est aussi connu comme un des agents microbiens causant de plus des maladies liées à la mammite bovine. La mammite consiste en une inflammation de la glande mammaire, généralement développée en réponse à une infection bactérienne intra mammaire. Enfin, la mammite se reconnait aussi comme étant la maladie la plus importante rencontrée au sein des troupeaux laitiers. *S. aureus* revêt dans ce sens une importance particulière, car il est très infectieux et se caractérise par des taux de guérison significativement inférieurs par rapport aux infections causées par d'autres microorganismes. L'objectif majeur de cette thèse est d'aborder les mécanismes favorisant les phénomènes de résistance rencontrés chez les bactéries de *S. aureus* en utilisant un ensemble de tests génotypiques et phénotypiques, du *S. aureus* isolé des mammites dans le nord-ouest du Portugal. Ainsi, dans la première partie de cette thèse, nous nous intéressons à l'étude de l'activité antimicrobienne des bêta-lactamines et de la diversité du gène *blaZ* dans les isolats de *S. aureus* de mammite bovine d'origine nord-ouest du Portugal afin de montrer les mécanismes par lesquels le *S. aureus* peut conférer une résistance aux antibiotiques de la famille bêta-lactamines et ce malgré la présence d'une grande diversité de différents groupes d'antibiotiques au sein de cette famille. En général, cette première étude visait à évaluer la sensibilité antimicrobienne de 52 isolats de mammite à *S. aureus* obtenus dans les années 2003-2004, 2007-2008 et 2017 dans le nord-ouest du Portugal contre des antibiotiques appartenant à la famille des bêta-lactamines; et de prévoir la prévalence, détecter et étudier la diversité des gènes de résistance *blaZ* et *mecA* dans les isolats positifs était aussi un des objectifs principaux de cette étude.

D'après les résultats obtenus des isolats collectés à partir de l'antibiogramme au cours des périodes 2003-2004 et 2007-2008, nous avons trouvé que la résistance à l'aztréonam (groupe Monobactames) a été observée dans tous les isolats (100.0%), alors que la résistance à la

pénicilline (groupe Penicillines G) était considérablement plus élevée avec 76.9%, suivie par une résistance de 73.0% à l'ampécilline (groupe Aminopenicillins). La résistance à l'amoxicilline et à l'oxacilline n'était que de 3.8%, tandis que la sensibilité à l'amoxicilline combinée à l'amoxicilline/acide flavulanique était 100.0% (tous du groupe Aminopenicillins). Aussi, toutes les souches ont présenté une sensibilité de 100.0% aux autres antibiotiques étudiés parallèlement comme la céfazoline (groupe des céphalosporines), la pipéracilline (groupe des uréidopénicillines), l'imipénem (groupe des carbapénems) et la ticarcilline (groupe des carboxypénicillines). Cependant, les résultats obtenus par le biais de réactions de PCR ont montré que tous les isolats testés dans cette étude étaient positifs pour le gène de *blaZ* et un seul isolat qui a seulement été trouvé positif au gène du *mecA*. L'analyse phylogénétique du gène de *blaZ* détecté a placé les isolats dans 3 groupes différents qui sont étroitement liés à d'autres souches différentes de mammite bovine et de *S. aureus* d'origine humaine. Cependant, les nouveaux résultats obtenus à partir des nouvelles souches collectées en 2017 ont montré une nouvelle prévalence pour tous les antibiotiques testés tels que la pénicilline (27.0%), l'ampicilline (34.6%), l'oxacilline (65.4%), l'amoxicilline combinée à l'acide clavulanique (34.6%) et à la céfazoline (42.3%). De plus, un taux de résistance intermédiaire de 11.5%, 3.8%, 3.8%, 3.8%, 19.2% et 3.8%, respectivement, a été observé pour ces antibiotiques déjà décrits. Enfin, l'antibiotique pipéracilline a toujours montré une sensibilité permanente de 100.0% contre toutes ces nouvelles souches collectées. La comparaison de ces nouveaux résultats avec ceux des périodes 2003-2005 et 2007-2008 est très différente. Ainsi, nous avons pu observer une réduction notable, surtout pour la pénicilline (27.0% en 2017 au lieu de 76.9% pour la période de 2003 à 2008) et pour l'ampécilline (34.6% en 2017 au lieu de 73.0% en 2003 à 2008). En conclusion de cette première partie de cette thèse ; nous avons observé, en particulier dans le cas des isolats collectés de 2003 à 2008, une prévalence de résistance élevée dans les souches de *S. aureus* testés notamment contre la pénicilline et l'ampécilline alors que les autres groupes d'antibiotiques testés, au sein de la famille des bêta-lactamines, ont montré une activité atteignant les 100.0% contre cette bactérie pathogène de *S. aureus*. Parallèlement, une prévalence de 100.0% a été observée pour le *blaZ*, gène codant la résistance aux bêta-lactamines. Cependant, l'analyse phylogénétique a placé les isolats dans trois groupes étroitement liés à différentes souches de mammite bovine et de souches de *S. aureus* d'origine humain, l'isolat 2 étant le plus divergent. Dans les groupes 1 et 3, la plupart des isolats sont liés à une forte prévalence de la résistance à la pénicilline et à l'ampécilline. En ce qui concerne les isolats présentant une susceptibilité phénotypique de 100.0%, ils sont généralement inclus dans le groupe/sous-groupe 1 et le groupe 2. Cependant nous avons, dans le cas des nouvelles

souches collectées en 2017, noté une diminution de la prévalence de la résistance aux deux antibiotiques : pénicilline et ampécilline. Ceci pourrait être par exemple expliqué par les pratiques vétérinaires; car, en raison de la forte prévalence de résistance de *S. aureus* à la pénicilline et à l'ampécilline à travers le monde, de nombreux vétérinaires ont choisi d'éviter autant que possible ou définitivement l'utilisation et la prescription de ces deux antibiotiques bien connus dans le traitement des infections bactériennes, notamment pour les mammites causées par *S. aureus* en vue de la mauvaise réputation qui caractérise la famille des Bêta-lactamines dans le monde à cause du phénomène de résistance. Ainsi, le fait d'éviter l'utilisation de ces antibiotiques dans les pratiques thérapeutiques, pour un temps assez long, pourrait conduire à l'inactivation des gènes responsables (tels que *blaZ*, *mecA*) de l'acquision de résistance du *S. aureus* à ces deux antibiotiques très connus en médecine. De plus, nous avons aussi observé l'apparition de souches résistantes intermédiaires au sein des nouvelles souches collectées durant 2017 d'une part, et l'apparition d'isolats présentant une résistance à un certain nombre d'antibiotiques comme l'amoxicilline combinée à l'acide clavulanique et la céfazoline, qui étaient 100.0% plus efficaces contre les isolats recueillis entre 2003 et 2008, d'autre part. Enfin, l'antibiotique pipéracilline a gardé son efficacité contre les souches plus anciennes collectées entre 2003 et 2008, et il s'avère également très efficace contre les nouvelles souches qui ont été récemment collectées en 2017. Il faut donc reconsidérer cet antibiotique pour combattre et traiter les infections à *S. aueus* provenant de la mammite bovine *in vivo*. La nouvelle analyse phylogénétique du *blaZ* de toutes les souches de *S. aureus* collectées au cours des années 2003, 2004, 2007, 2008 et 2017 a montré la diversité à l'intérieur ou entre différents troupeaux du nord-ouest du Portugal.

Dans la deuxième partie de cette thèse, nous avons essayé d'élargir notre étude pour inclure d'autres antibiotiques d'autres familles afin d'avoir une vision globale sur les profils de résistance de *S. aureus* issus de la mammite bovine dans le nord-ouest du Portugal. Ainsi, l'objectif de cette deuxième partie était de détecter certains gènes codant pour la résistance du *S. aureus* d'une part et d'étudier la prévalence et d'évaluer in vitro l'efficacité d'un ensemble d'antibiotiques contre les souches du *S. aureus* isolées à partir de la mammite bovine dans le nord-ouest du Portugal d'autre part. Pour cette raison, les tests de sensibilité aux antimicrobiens ont également été effectués par la méthode de diffusion sur disque, tandis que la détection des gènes tels que les enzymes modifiant les aminoglycosides (*aph(3')-III-a*, *ant(4)-I-a* et *aac(6')-aph(2')*), *tetM*, *tetK*, *ermT*, *vanA*, *spc*, *lnuC*, *salA*, *vgaC* et *dfrK*, était également réalisée par des méthodes spécifiques PCRs.

D'après les résultats obtenus lors de cette étude, nous avons trouvé que sur les 52 isolats de *S. aureus* testés, 39 (75.0%), 35 (67.3%), 26 (50.0%), 22 (42.3%), 19 (36.5%), 17 (32.7%), 15 (28.8%), 12 (23.1%) et 11 (21.2%) ont présenté une résistance élevée plus particulièrement à la tétracycline, à l'érythromycine, à la vancomycine, à l'amikacine, à la kanamycine, à la clindamycine, à la gentamycine, à la tobramycine et au trimetroprim-sulfatomexazole, respectivement. En revanche, 18 (34.0%), 15 (28.8%), 14 (26.9%), 11 (21.2%), 7 (13.5% chacun) et 4 (7.7% chacun) ont présenté une résistance intermédiaire à la vancomycine, au triméthroprim-sulfatomexazole, à la tobramycine, à la gentamycine, à la tétracycline, à l'érythromycine, à la kanamycine, à la clindamycine et à l'amikacine. Parmi les gènes de dépistage, *ant(4)-I-a*, *tetM*, *aph(3')-II I-a*, *dfrK*, *tetK*, *lnuC* et *spc* ont été détectés dans 33 (63.5%), 30 (57.7%), 16 (30.8%), 10 (19.2%), 8 (15.4%), 3 (5.8%) et 1 (1.9%), respectivement. De plus, les gènes testés restants tels que *vanA*, *ermT*, *salA*, *vgaC*, *aac (6') - aph (2')* étaient tous négatifs. Tous les antibiotiques utilisés dans cette étude ont montré une efficacité remarquable et élevée sur toutes les souches collectées principalement entre 2003 et 2008. Alors que toutes les nouvelles souches collectées plus récemment en 2017 étaient, dans la plupart des cas, résistantes ou intermédiaires contre tous ces antibiotiques, montrant ainsi une évolution alarmante et dramatique de l'apparition de nouvelles souches très résistantes conduisant à repenser immédiatement voire à changer définitivement notre vision pour l'utilisation de ces antibiotiques avant que l'humanité déclare une capitulation totale aux nouvelles infections dues à *S. aureus* dans les quelques prochaines années.

En conclusion de cette deuxième partie de cette thèse, nos résultats illustrent principallement la présence d'une forte prévalence de plus de 50.0% de résistance phénotypique dans les isolats de *S. aureus* testés contre la tétracycline, l'érythromycine et aussi contre la vancomycine. De plus, une prévalence élevée de 63.5% et 57.7% a été détectée, par PCR, pour *ant(4)-I-a* et *tetM*, respectivement. D'autre part, nous avons observé dans quelques cas que les résultats obtenus ont confirmé que la proportion d'isolats de *S. aureus* présentant une résistance phénotypique ne correspondait pas à celle des isolats identifiés avec les gènes détectés à savoir *blaZ*, *ermT*, *tet* les enzymes modifiant des Aminoglycosides gènes car par exemple certaines souches de *S. aureus* présentant 100.0% de sensibilité phénotypique pour tous les antibiotiques testés portaient également le gène *blaZ*. Des résultats équivalents ont été observés pour les gènes *ermT* et *tet,* les gènes des enzymes modificateurs des Aminoglycosides. L'explication de ce phénomène est trop détaillée dans la partie de discussion. Enfin, les résultats présentés dans cette étude pourraient être utiles pour concevoir des programmes spécifiques de lutte contre la mammite bovine causée par *S. aureus* dans la région nord-ouest du Portugal.

List of Abbreviations

AKN	Amikacin
AMX	Amoxicillin
AMC	Amoxicillin + Clavulanic Acid
AMEs	Aminoglycoside-Modifying Enzymes
AMP	Ampecillin
AMPs	Antimicrobial Peptides
AZT	Aztreonam
BHI	Brain Heart Infusion
BTSCC	Bulk Tank Somatic Cell Count
C1G	First-Generation Cephalosporins
C2G	Second-Generation Cephalosporins
C3G	Third-Generation Cephalosporins
CA	Community-Associated
CA-MRSA	Community Associated- MRSA
CDCP	Centers for Disease Control and Prevention
CFUs	Colony Forming Units
CFZ	Cefazolin
CL	Chloramphenicol
CLDM	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
CMT	Califorina Mastitis Test
CNS	Coagulase-Negative Staphylococci
CPS	Coagulase-Positive Staphylococci
CRF	Coagulase-reacting factor
CSF	Colony-Stimulating Factors
cSSSI	complicated Skin and Skin Structure Infections
DNA	Deoxyribonucleic acid

dNTPs	deoxynucleotides
DSPs	Disaccharidepentapeptides
EARSS	European Antimicrobial Resistant Surveillance System
EBVM	Evidence Based Veterinary Medicine
ECM	Extracellular Matrix
eDNA	Environnement DeoxyriboNucleic acid
EDTA	Ethylene Diamine Triacetic Acid
ERY	Erythromycin
EMA	European Medicines Agency
ETs	Exfoliative Toxins
FAO	Food and Agriculture Organization
FAOUN	Food and Agriculture Organization of the United Nations
FC	Fibrinogen receptor
G7	An economic group closing seven countries
G20	An economic group closing twenty countries
GAP	Global Action Plan
$G^{+}C$	Gram positive
GDP	Gross Domestic Product
GEN	Gentamicin
HA	Health care-Associated
HA-MRSA	Hospital-Acquired-MRSA
HPRA	Health Products Regulatory Authority
ICAM-1	Intercellular Adhesion Molecule 1
ICBAS	Institute of Sciences Biomedical Abel Salazar
IFN-g	Interferon gamma
Ig	Immunoglobulin
IL-1b	Interleukin 1 beta
IMI	Intramammary Infection
IMP	Imipenem
KAN	Kanamycin

LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MBL	Mannose-Binding Lectins
MDR	Multidrug Resistance
MHCII	Major Histocompatibility Complex Class II
MIC	Minimum Inhibitory Concentration
min	Minute
ML	Maximum Likelihood
MLS	Macrolide, Lincosamide and Streptogramin
MLSB	Macrolide-Lincosamide-Streptogramin B
mRNAs	messenger RiboNucleic Acid
MRSA	Methicillin-Resistant *S. aureus*
MSCRAMMs	Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-Susceptible *S. aureus*
NAG	N-acetylglucosamine
NAM	N-acetyl muramic acid
NEO	Neomycin
NF-kB	Nuclear Factor-kB
NK	Natural Killer cells
NOVO	Novobiocyn
OIE	World Organisation for Animal Health
OXA	Oxacillin
PAMPs	Pathogen-Associated Molecular Patterns
PBP2a	Penicillin-Binding Protein 2a
PBPs	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
PEN	Penicillin
PGN	Peptidoglycan
PIP	Piperacillin
PLX	Polymixine

PRAC	Pharmacovigilance Risk Assessment Committee
PVL	Panton-Valentine Leukocidin
RNA	Ribonucleic Acid
rRNA	ribosomal RiboNucleic Acid
S	Second
SCC	Somatic Cells Count
STN	Streptomycin
TBE	Tris Borate Edta
TMN	Tobramycin
TET	Tetracycline
TIC	Ticarcillin
TLRs	Tolllike Receptors
TMP/SMX	Trimethoprim/sulfamethoxazole
TNF-a	Tumor Necrosis Factor alpha
TSS	Toxic Shock Syndrome
TSST-I	Toxic Shock Syndrome Toxin I
UK	United Kingdom
US	United States
USA	United State American
USD	United State Dollar
UV	Ultra Violet
VANCO	Vancomycin
β-lactam	Beta-lactams
$	Dollar

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General Introduction

In veterinary and human medicine, antimicrobial agents play an important role in the therapy of bacterial infections (**Wendlandt *et al*., 2015**). However, the resistance problem that human and animal health encountered often after use of these antimicrobial agents against these bacterial infections manifest, often quickly, after each introduction of new classes of antimicrobial drugs (**Knobler *et al*., 2003**). The discovery of antimicrobial agents during the 20th century has been considered one of the wonder discoveries (**Davies & Davies, 2010**), but the increase of antimicrobials resistance encountered in hospitals, communities, and the environment concomitant with their utilization also increased (**Davies & Davies, 2010**).

Mastitis consists of an inflammation of the mammary gland, usually developed in response to an intramammary bacterial infection (**Seegers *et al.,* 2003; Olsen *et al.,* 2006, Mansor *et al.,* 2013**). Worldwide, bovine mastitis is considered to be a common disease, touching dairy cows with high incidence (**Gomes *et al*., 2016**). They are considered the most expensive factor in the dairy industry (**Gomes *et al*., 2016**) and also limit the profitability of the dairy farm (**Ruegg *et al*., 2015**). The classification of mastitis can be referred into three major types: clinical mastitis, sub-clinical mastitis (**Gruet *et al*., 2001; Anonymous, 2003; Awale *et al*., 2012**) and chronic mastitis (**Anonymous, 2003; Hassan *et al.,* 2016**). Sub-clinical mastitis type being the form the most frequently encountered in dairy herds (**Gruet *et al*., 2001**; **Awale *et al*., 2012**). In clinical mastitis form, there is swelling, pain, heat, and indurations observed in the mammary gland and also changes in colour of milk, clots are present in the milk and there are large leukocytes numbers in the milk (**Sharma & Maiti, 2010**). However; in sub-clinical mastitis form, there are no gross inflammatory changes in the udder tissue and no changes physical appearance on milk. Only increased somatic cell count in the milk, the presence of pathogenic organisms in the milk, and an inflammatory response that can only be detected by screening tests such as California Mastitis Test, White Side Test, Surf Field Mastitis Test (**Sachin & Suresh, 2006; Madut *et al*., 2009**). In case of chronic mastitis form, udder becomes hard due to fibrosis and the quarters may become thickened, firm, nodular and atrophic. The milk can appear as yellowish fluid or white with clots and flakes. Infrequently, it can look as green or yellow green and foul smelling (**Chakrabarti, 2007; Hassan *et al.,* 2016**).

Staphylococcus aureus (*S. aureus*) is a Gram positive bacteria considered to be an important human pathogen and known as one of the most important agent associated with bovine mastitis worldwide (**Olsen *et al.,* 2006**). In general, the *S. aureus* is known as a bacterium that can employ galactose and lactose as energy sources, and as such is a notorious cause manifested in case of bovine and ovine mastitis (**O'Reilly *et al.,* 1992; Almeida *et al.,* 1996**) and food poisoning via contaminated food, dairy associated especially. Staphylococci are considered to

be together with Streptococci members of a group of bacteria recognized as the invasive pyogenic cocci, since they can cause a variety of suppurative or pus-forming diseases in humans and other animals (**Moreillon *et al.,* 2005**). Indeed, more than 90.0% of clinical *S. aureus* isolates elaborate polysaccharidic capsules, among which 11 serotypes have been showed (**Sompolinsky *et al.,* 1985; Luong *et al.,* 2002**). According to authors, capsules type 5 and type 8 *S. aureus* have been encountred in up to 75.0% of clinical infections caused by this pathogenic bacterium. However, around of 20.0% of human isolates and up to 86.0% of bovine strains of *S. aureus* have been so far non-typable (**Cocchiaro *et al.,* 2006**). As well, the capsule protects the bacteria from phagocytosis. Lysozyme, is a muramidase enzyme that present in various host tissues such as mucous membranes, respiratory and intestinal tracts and also in fluids such as serum, saliva, and tears do not kill *S. aureus*. This resistance can contribute to its capacity to colonize skin and mucosal tissues, for example, the anterior nares (**Bera *et al.,* 2006**). Mechanisms of persistence of *S. aureus* in intra-mammary environments still need to be explored but evasion of host immune system and adherence to epithelial cells of mammary glands are some of the known in this regard (**Almeida *et al.,* 1996**).

Bovine mastitis, caused by staphylococcal or other agents, are recognized as an endemic disease and considered to be the most prevalent and expensive disease in the dairy farms, still remaining as an economically relevant problem to the dairy industry in several countries (**Barkema *et al.,* 2006**; **Halasa *et al.,* 2007**). About 140 microorganisms species, have been recognized as etiological agents of bovine mastitis (**Watts, 1988**), as *Klebsiella* spp., *Pseudomonas aeruginosa*., *S. uberis*., *E. coli* and pyogenic bacteria appeared associated with clinical mastitis, while others microorganisms like *S. agalactiae*, coagulase-negative staphylococci and *Enterococcus* spp. appear related well with the subclinical mastitis form (**Bradley, 2002**; **Barkema, 2009**; **Awale *et* 2012**). In dairy cows, the sub-clinical type of mastitis is considered to be more important because it is 15 to 40 times more prevalent than the clinical form on the one hand as it usually precedes the clinical form on the one other hand. As well, this form is well-known of its long duration, difficulty to detect, reduction milk production, and of it's adversely affectation milk quality (**Seegers *et al.*, 2003**). In general, the sub-clinical form mastitis is more serious and it is responsible for much greater loss to the dairy industry in many countries (**Kader *et al.*, 2003**). *S. aureus* is of particular importance, because it is highly infectious (**Kerro *et al.,* 2002**) and it is characterized by significantly lower cure levels in comparison with infections caused by other microorganisms (**Cramton *et al.,* 1999**). Moreover, the *S. aureus* has the potential to expand resistance to almost all the antimicrobial agents (**Hiramatsu *et al.,* 2001; Barkema *et al.,* 2009**). In herds, ~80.0% of antimicrobials are

reported for the treatment of bovine mastitis (**Zwald *et al*., 2004**), as antibiotic therapy remains the primary choice for mastitis control in lactating and dry cows (**Rajala-Schultz *et al*., 2004**). The first antimicrobials usage in veterinary medicine was noted in dairy cows for treatment mastitis disease (**Mitchell *et al.,* 1998**).

Antimicrobials are defined as substances that destroy or inhibit the growth of pathogenic groups of microorganisms, including bacteria, viruses, protozoa, and fungi, while antibiotics are defined as substances (not limited to those produced from microorganisms) that can kill or inhibit the growth of bacteria (**Stacey *et al.*, 2003**). Microorganisms remained as one of the most important production sources of new bioactive substances including antibiotics, immune-suppressants, anti-parasitics, anti-tumor, hypocholesterolemic agents, and enzyme inhibitors (**Chen *et al.,* 2009; Sumi *et al.*, 2014; Demain, 2014**).

In veterinary medicine, antibacterials remain the most important drugs administered (**Dasenaki & Thomaidis, 2017**). According to authors, the classifications of antibacterial are based on their mechanism of action, chemical structure, and spectrum of activity or source. The most important classes are Beta-lactams (Cephalosporins and Penicillins), Aminoglycosides, Macrolides, Lincosamides, Amphenicols, Nitrofurans, Quinolones, Sulfonamides, Tetracyclines, and Miscellaneous (**Dasenaki & Thomaidis, 2017**).

Beta-lactam antibiotics family, such as penicillin compounds, continues to be one of the most frequently used drugs in veterinary and human medicine (**Pitkala *et al.,* 2007**). Indeed, Beta-lactams have been the most widely used antibiotics drugs for more than eight decades and still constitute the most important group of antibiotics described in medicine. They are divided into two subcategories: Penicillins and Cephalosporins. In general, Beta-lactam family is used as growth promoters, and chemotherapeutic and/or prophylactic agents; but, their extensive utilization in veterinary medicine practices causes numerous residues in foodstuffs, which present a serious health hazard, mainly regarding the development of resistance in target organisms (**Lara *et al.,* 2012**). In addition to this antimicrobials family, the discovery of Aminoglycosides compounds also introduced a new therapeutic concept in medicine, especially after the discovery and emergence of the first penicillin resistant strains after the introduction of this antibiotic for the first time in 1940, what constitutes a new challenger that has really started to threat human and animal health. In 1943, the discovery by Selman Waksman and his student, Albert Schatz, of streptomycine, a natural antibiotic molecule produced by *Streptomyces griseus* (**Schatz *et al.,* 1944**), caused great excitement in the medical community as it proved to be the first clinical treatment against tuberculosis. This earned Waksman the Nobel Prize for Medicine in 1952. In the years following this landmark success, many similar

compounds, such as neomycin or kanamycin, were isolated from bacterial strains of the family Actinomycetes (**Walsh, 2003**). Aminoglycosides compounds family are antibacterials with broad-spectrum isolated from Streptomyces and Micromonospora bacteria. This antibiotics family compounds are characterized by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component (**McGlinchey *et al.,* 2008**), and they are administered both therapeutically and prophylactically to treat cattle, swine, and poultry (**Dasenaki & Thomaidis, 2017**). In the literature, Macrolides were the third major class of microbial products to be discovered that possess antibiotic properties after Beta-lactams and Aminoglycosides (**Lewis, 2013**). Macrolides are antibiotic compounds composed of 14 (erythromycin and clarithromycin), 15 (azithromycin), or 16 (josamycin, spiramycin, tylosin)-membered lactones to which amino and/or neutral sugars are linked (**Roberts *et al.,* 1999; Roberts, 2008, Iannelli *et al.,* 2018**). The archetypal Macrolide, erythromycin, was the first antibiotic compound isolated from the soil dwelling bacterium *Saccharopolyspora erythrea* in 1949 in a Filipino environmental sample (**Golkar *et al.,* 2018**). Three years later, this Macrolide antibiotic entered clinical practice, exactly in 1952. According to **Golkar *et al.,* 2018**, this kick-started the golden age of Macrolide discovery where a plethora of new Macrolides were being frequently characterized. What is more interesting, this marvellous discovery fueled the development of next-generation Macrolides using semi-synthetic approaches (**Bryskier, 2000**). In general, Macrolide antibiotics have been used and described in both agriculture and medicine (**Golkar *et al.,* 2018**). Furthermore, Macrolides such as erythromycin and azithromycin have been used as substitutes for Beta-lactam antibiotics in patients with penicillin allergies (**MacLaughlin *et al.,* 2000; Golkar *et al.,* 2018**). In the world, azithromycin remains among the most successful and highly prescribed antibiotics (**Andrew *et al.,* 2018**). In medicine, the great neediness for an alternative treatment to penicillin-allergic patients has increased the clinical application of Macrolides worldwide **(Golkar *et al.,* 2018**). Furthermore, Macrolide, Lincosamide and Streptogramin B (MLSB) antibiotics stay one of the available options for treating infections caused by *Staphylococcacea* germs (**Saribas *et al.,* 2006**). Action mode of MLSB antibiotics are similar because they inhibit synthesis of protein by targeting the peptidyl transferase center within the 50S subunit (23s rRNA) of the bacterial ribosome (**Bozdogan & Appelbaum, 2004**). In this context, the peptidyl transferase center is known as the main target site for many antibiotics, but the exact mechanism for its activity is still unclear (**Schlünzen *et al.,* 2001**). Several studies suggested that MLSB could also inhibit peptidyl transferase by interfering with the proper positioning and movement of the tRNAs at the peptidyl transferase cavity (**Franklin & Snow, 1975; Brisson-Noël *et al.,* 1988; Petinaki & Papagiannitsis, 2018**).

The discovery of Tetracyclines was also in the 1940s, Tetracyclines are an antibiotics family that inhibits synthesis of protein by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (**Chopra & Roberts, 2001**). Otherwise, Tetracycline inhibits cell growth by binding to the 16S part of the 30S ribosomal subunit preventing aminoacyl tRNA from binding to the ribosome A site. This leads to inhibition of translation hampering cell growth (**Connell *et al.,* 2003**). The wide use of Tetracyclines, in veterinary medicine, was for cost-effective prophylactic and therapeutic treatment and also as growth promoters in cattle and poultry (**Dasenaki & Thomaidis, 2017**). In general, Tetracyclines are broad-spectrum compounds, exhibiting activity against a large variety of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (**Chopra & Roberts, 2001**). The favourable antimicrobial properties of Tetracyclines compounds and the absence of major adverse side effects have conducted to their widespread utilization in the therapy of human and animal infections (**Chopra & Roberts, 2001**). Chloramphenicol is else an antibiotic with broad spectrum, it was originally isolated from culture filtrates of the fungus *Streptomyces venezuelae* (**Ehrlich *et al.,* 1947; Ehrlich *et al.,* 1948; Gruhzit *et al.,* 1949; McGhee & Anastas, 1996; Erel *et al.,* 1999**) by Bartz (**Bartz, 1948**) and subsequently was synthesized chemically (**Controulis *et al.,*1949; Gruhzit *et al.,* 1949**). Chloramphenicol acts by disrupting bacterial peptide bond formation by reversibly binding to the 50s subunit of the 70s ribosome, inhibiting the formation of bacterial ribosomes from soluble RNA (**McGhee & Anastas, 1996; Erel *et al.,* 1999**). On the whole, Aminoglycosides, Macrolides, Lincosamides, Streptogramins, Chloramphenicols and Tetracyclines family are antibiotics that act as protein synthesis inhibitors. However, and because of importance of exchange of intra- and extracellular substances that takes places through microbial cell membranes. Certain anitimicrobial agents act as cell membrane function inhibitors such as Polymyxin, a group of antibiotics that is characterized by a cyclic peptide with a long hydrophobic tail (**Das & Patra, 2017**). Thus cell survival can be at stake if there is disruption of cell membrane structure because of leakage of important intercellular solutes. Cell membrane is found in both prokaryotic and eukaryotic organisms causing poor selectivity and thus compromising its use in the mammalian host, for example, Polymyxin (**Das & Patra, 2017**). In human medicine, the administration of synthetic peptides as therapeutics or diagnostics is well-established; but, a few of these drugs have also found use for comparable indications in veterinary. Peptide-based drugs are especially indicated for treating animals used in food production, though regulation of fertility is their most important application.

In parallel to utilization of antibacterials originated from microorganisms, there are also introduction of synthetic antibacterial compounds such as Sulfonamides, Nitrofurans and Quinolones in treatment of several diseases infections caused by pathogenic germs. For example, Sulfonamides (sulphonamides) are a group of man-made (synthetic) medicines that contain the Sulfonamide chemical group. They are also known as Sulfa drugs name. The first Sulfonamide developed was sulfanilamide in 1906, but it was not utilised as an antimicrobial compound until the late 1930s (**Fookes, 2018**). Sulfonamides have a broad-spectrum bacteriostatic (stop bacteria from reproducing but don't necessarily kill them) of antimicrobial activity against many microorganisms including bacteria and some protozoa, such as Toxoplasma and Plasmodia (**Scholar, 2017**) and work by interfering with the synthesis of folic acid in bacteria, which is essential for nucleic acid formation and ultimately RNA and DNA (**Fookes, 2018**). Humans achieve folic acid from their diet, although bacteria need to synthesize it. Sulfonamide antimicrobials can be combined with trimethoprim to make them bactericidal, because trimethoprim acts on a different enzyme in the folic acid synthesis pathway (**Fookes, 2018**). The Nitrofuran antimicrobials like nifurtimox and furazolidone are known for their therapeutic value in the treatment of several illnesses, caused by protozoa or by certain gram-positive or gram-negative bacteria (**Dasenaki & Thomaidis, 2017**), such as trypanosomiasis, giardiasis and urinary tract infections in humans **(Sharma & Anand, 1997)** and do not contribute to the development of antimicrobial resistance (**Dasenaki & Thomaidis, 2017**). In the European Union, the use of Nitrofurans for livestock production was completely prohibited in 1995, due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health (**Vass *et al.*, 2008; Dasenaki & Thomaidis, 2017**). However, Quinolones and Fluoroquinolones are known as a class of broad-spectrum antibiotics that are active against Gram-negative and Gram-positive bacteria (**EMA, 2018**). For what is concerning their use nowadays in medicine, the European Medicines Agency's (EMA) Pharmacovigilance Risk Assessment Committee (PRAC) has recently recommended restricting the use of fluoroquinolone and quinolone antibiotics (described by mouth, injection or inhalation) following a review of disabling and potentially long-lasting side effects reported with these drugs. The review incorporated the views of patients, healthcare professionals and academics presented at EMA's public hearing on Fluoroquinolone and Quinolone antibiotics in June 2018 (**EMA, 2018**). Except for the antibacterials belonging in the precedent groups, there are also several subgroups of antibacterials, such as Pleuromutilins, Diaminopyrimidines, Peptides, Quinoxalines, and Dapsone, which are widely used in meat producing animals. Pleuromutilins were discovered as natural-product antibiotics in 1950. However, the appearance of tiamulin as

the first Pleuromutilin compound to be approved for veterinary use was in 1979, followed by valnemulin in 1999. Likewise; we must wait till 2007, date that retapamulin became the first Pleuromutilin approved for use in humans (**Novak & Shlaes, 2010**). All antibiotics from Pleuromutilins class are generally known as compounds that selectively inhibit bacterial translation and are semisynthetic derivatives of the naturally occurring tricyclic diterpenoid pleuromutilin, which received its name from the pleuromutilin-producing fungus *Pleurotus mutilus* Tiamulin and valnemulin are two established derivatives in veterinary medicine for oral and intramuscular administration (**Paukner & Riedl, 2017**). Concerning antibacterial Diaminopyrimidines class, it is well notorious that the value of the Sulfonamides as single antimicrobial agents has been greatly diminished both by their relatively low potency compared to more modern antimicrobial drugs on the one hand and by widespread acquired resistance on the one other hand. Conversely, when combined with antibacterial Diaminopyrimidines such as trimethoprim, resistance occurs less frequently and thus their usefulness has been enhanced (**Prescott, 2013**). Quinoxaline moiety is a part of various antibiotics such as echinomycin, levomycine, actinoleutine and also acts as antiviral, anti-diabetic, anti-inflammatory, kinase inhibitors, anticancer, ion channel regulators and anti-protozoa agent. Although, Dapsone is a chemical class different from Sulfonamides but its mechanism of action is similar to Sulfonamides via inhibition of bacterial synthesis of dihydrofolic acid by competing with para-aminobenzoate for the active site of dihydropteroate synthase. There is no veterinary approved form of Dapsone. It is potentially useful for the oral treatment of some protozoal infections in horses. Dapsone class is carcinogenic and should be used with caution in pregnant and nursing animals.

Staphylococcus aureus, Streptococcus pneumoniae, and enterococci are well-known to be among various pathogenic gram-positive bacteria and they stand out as being responsible for global resistance challenges, significant public health burden, and cost to healthcare (**Woodford & Livermore, 2009**). In human as in animal health, Gram-positive infections can result in a wide range of diseases, including mastitis, osteomyelitis, endocarditis, thrombophlebitis, meningitis, skin and soft tissue infections, surgical and trauma wound infections, urinary tract infections, gastrointestinal tract infections, pneumonia, toxic shock syndrome, septicaemia, and infections of indwelling medical devices (**Nair *et al.,* 2014**). Earlier, most infections caused by *Staphylococcae* species can be easily treated with antibiotics; however, in recent years *Staphylococcus* found its manner to resist the commonly used and effective antibiotics; these antibiotics include Macrolides, Lincosamides, Streptogramins, Tetracyclines, Aminoglycosides (particularly gentamicin) and Beta-lactams (particularly

methicillin) (**Gould *et al.,* 2005, Delorme *et al.,* 2009; Klein *et al.,* 2013; Shen *et al.,* 2013**). These antibiotics families are extensively used and more described in veterinary medicine for cost-effective prophylactic and therapeutic treatment and they are also used as growth promoters in cattle and poultry. However, their usefulness has been reduced with the onset of bacterial resistance (**O¨nal, 2011**). For example, the large Tetracyclines description in veterinary medicine was used mainly for cost-effective prophylactic and therapeutic treatment and also as growth promoters in cattle and poultry (**Dasenaki & Thomaidis, 2017**). The importance of antimicrobial therapy in dairy cow stays an imperative strategy for mastitis control as well as human infections (**Gomes & Henriques, 2016**). On the other hand, *S. aureus* often show evidence of resistance to multiple antimicrobial compounds classes as a response to the selective pressure of antimicrobials, which will narrow the treatment options for veterinarians and clinicians (**Gomes & Henriques, 2016**).

The occurrence of mastitis pathogens and their antimicrobial resistance have been studied in numerous scientific investigations around the world (**Kalmus *et al.,* 2011**). Concerning the evolution of this resistance prevalence in the world, for example **Erskine et *al.,* (2001)** have concluded in a retrospective study on mastitis malady in Finland two times the reduction of *S. aureus* resistance against penicillin while six times resistance against erythromycin over a period of 6 years. According to **Aqib *et al.,* (2019)**, this was not true because in reports encompassing findings of studies conducted in other geographical zones where resistance to the antibacterial drug increased to double of what was reported 12 years ago (**Myllys *et al.,* 1998; Pitkala *et al.,* 2001).** Later to 2001, the studies have indicated increase in general resistance of *S. aureus* strains against antibiotics compounds. According to **Aqib *et al.,* (2019)**, this difference observed in trends could be attributed to evolution of resistance against local microflora being under therapy selection, drug regulation of country, traditions of farmers, local antibiotic therapy protocols, and number of processed samples in the study.

To date, antibiotics drugs have been developed in order to fight against almost all pathogenic bacteria causing various infections and are now readily available worldly. According to **Lewies *et al.,* (2019)**, the successful deployment of antibiotics has unfortunately resulted in these drugs being used more as a financial commodity rather than a valuable community resource that should be rationally managed. This has conducted to the accelerated development of antimicrobial resistance among many bacteria over the world (**Lewies *et al.,* 2019**). However, the growing resistance noted to antibiotics drugs can be connected to antibiotic overuse and requires to be addressed promptly as previously reported by **Netsvyetayeva *et al.,* (2014)**. It appears from the literature reports that the extensive use of antibiotics in humans as well as

their employ for disease prevention and growth promotion in agriculture has led to the appearance of antibiotic-resistant strains (**Hamer & Gill, 2002; Martínez & Baquerom, 2002; Phillips *et al.,* 2004; Ma *et al.,* 2018**).

Worldly, the evolution of the resistance problem will be aggravated over the decades. Particularly, the appearance of multi-drug-resistant (MDR) *S. aureus*, principally methicillin-resistant *S. aureus* (MRSA), leading to animal and human infections, has become an emergent public health concern (**Li *et al.,* 2015**). The first emergence of *S. aureus* strains that were resistant to methicillin was identified in the United Kingdom in 1961, soon after the introduction of methicillin antibiotic in medical practice in order to treat infections caused by penicillin resistant *S. aureus* (**Jevon, 1961, DeLeo & Chambers, 2009; Simonetti *et al.,* 2011; Kejela & Bacha, 2013**). According to **Frana *et al.,* (2013)**, around of 1.5% of the population (~4.1 million persons) is colonized with MRSA leading to at least 94,000 invasive infections and over 18,000 deaths annually only in the United States.

MRSA has appeared as a most important causative agent of health care-associated (HA) and community-associated (CA) infections (**Klein, 2007**). More recently, the spread to public health presented new strains entitled livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA). The frequent isolation of LA-MRSA has been showed by farmers, veterinarians, and farm workers' family members as previously reported by **Loncaric *et al.,* (2013**). In this regards, **Aqib *et al.,* (2019)** described that a clonal complex 398 representative of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) has proven the ability of colonization and serious health consequences in humans who are in close contact with animals.

MRSA strains have acquired a gene called *mecA* (this gene codes for a penicillin binding protein, PBP2a, which interferes with the effects of beta lactam antibiotics on cell walls) that makes them resistant to nearly all Beta-lactam antibiotics including semi-synthetic penicillins such as methicillin, oxacillin, or cloxacillin (Notable exceptions to this rule are the latest generation of cephalosporin Beta-lactams, for example, ceftaroline and ceftobiprole). Resistance to other antibiotics is also common, especially in hospital-associated MRSA (**CFSHP & IICAB, 2016**). However, *mecC*-bearing MRSA is a new resistance gene type of MRSA first recognized in 2011. Many of these organisms have been recovered from animals, particularly dairy cattle, but they can also infect and colonize humans. Therefore, *mecC* (formerly *mec*ALGA251) is also a Beta-lactams resistance gene, and is less well understood than *mecA* gene (**CFSHP & IICAB, 2016**). Similar to *mecA*, *mecC* gene is carried on SCCmec. This new gene codes for a different version of PBP2a, which is also thought to interfere with the effects of Beta-lactams drugs on cell walls. Nevertheless, a recent paper suggests that *mecC*-

encoded PBP2a may mediate resistance to some Beta-lactams antibiotics, but not others. This could raise the possibility of treatment with some drugs that are ineffective against *mecA*-bearing MRSA (**CFSHP & IICAB, 2016**). Nowadays, the MRSA strains are known as “a superbug” and they represent a major problem in most medical institutions because it is creating life-threatening situations (**Khan *et al.,* 2010**). Moreover, the manifestation of mutated strains of MRSA is the vancomycin resistant *S. aureus* (VRSA) which has added danger to health care communities. In the recent years, VRSA is really became one of the greatest threats-mankind faces because the antibiotic, vancomycin, is the last available alternative used for treating staphylococcal infections (**Khan *et al.,* 2010**).

Antibiotic resistance is now reaching dangerously high levels in all parts of the world. New resistance mechanisms are appearing and spreading around the world, compromising now our ability to treat common infectious diseases. For a growing number of infections, such as mastitis, pneumonia, tuberculosis, sepsis and gonorrhea, and food-borne illnesses, treatment becomes more difficult, if not impossible, because of the loss of antibiotic efficacy. Now, what is more dangerous remains the progressive evolution of increased resistance within pathogenic strains that has been almost noted against all kinds of available antimicrobials compounds and no introduction in parallel of any new drugs has invited the use of newer drug combinations (**Aqib *et al.,* 2019**). The use of combined antibiotics in medicine for treating many infections caused by resistance pathogenic bacteria has considered as one of the important strategies followed in recent years in order to stop resistance phenomenon and skip, therefore, this challenge implicated by these pathogenic microorganisms. Unfortunately, this strategy remains a temporary solution for many years because of the dramatically emergence of increased resistance that has been seriously threatening again many human and animal live around the world. For example, combination of cefaroxil from cephalosporins and amoxicillin from penicillins group showed synergistic effects against 80.0% of resistant isolates (**Aqib *et al.,* 2019**). During recent decades, Estonia was among countries where only antibiotics with broad-spectrum have been used for the treatment of clinical mastitis disease (**Kalmus *et al.,* 2011**). For example, in the years ranging from 2006 to 2009, 15 diverse combinations of antibiotics were on hand for use in 18 intramammary preparations that were authorised by the Estonian State Medical Agency (**ESMA, 2009**).

In the literature reports, the pathogenic resistance bacteria, particularly *S. aureus,* have nowadays developed and acquired several mechanisms allowing them to avoid efficacy of antimicrobials. For example, many genes such as *blaZ*, *mec*, *erm*, *aac/aph*, *tet*, *vga* and *van* are among the prevalent resistance genes noted to play a role in *S. aureus* resistance encoded for

several antibiotics such as Beta-lactams, Macrolides, Aminoglycosides, Tetracyclines and Glycopeptides families (**Vahaboglu *et al.,* 1998; Lina *et al.,* 1999; Martineau *et al.,* 2000; Strommenger *et al.,* 2003; Choi *et al.,* 2003; DeLeo & Chambers, 2009; Fabler *et al.,* 2010; Nizami *et al.,* 2012; Tang *et al.,* 2015; Farid *et al.,* 2015; Pekana & Green, 2018; Ma *et al.,* 2018**). The majority of *S. aureus* strains are now becoming multidrugs-resistance because of acquisition of several resistance genes encoding for almost of antimicrobials agents. Bacteria employ horizontal gene transfer from resistant to sensitive strains. Moreover, the development of resistance phenomenon to antimicrobial agents has been observed for a range of these described drugs above in addition to Fluoroquinolones (**Akpaka *et al.,* 2008**). As an example of resistance mechanisms encoded by genes, the inactivation of Beta-lactam antibacterial is effected by bacterial produced enzymes called Beta-lactamases (such as penicillinases, cephalosporinases, carbapenemases, cephamycinases, and so on). Concerning how Beta-lactam drugs work; according to **Bugg *et al.,* (2011**), Beta-lactam family acts as a false molecule for D-alanyl-D-alanyl transpeptidases, which result in inhibition of transpeptidation reaction and peptidoglycan synthesis. After that, autolytic enzyme inhibitors get inactivated, which activates the lytic enzyme, in that way resulting in division of bacteria provided that the environment is isotonic. Some other antibiotics such as vancomycin, novobiocin, bacitracin, teicoplanin and ristocetin must be subjected at early stages, which impede early phases of the peptidoglycan synthesis.

In countries where they are dispensed without a prescription for humans or animals, the problem of the emergence and spread of resistance is even worse. Similarly, in countries lacking standardized treatment guidelines, antibiotics are over-prescribed by health workers and veterinarians and over-consumed by the general public. If we do not take emergency measures, we will soon enter a postantibiotic era in which common infections and small wounds will again be fatal. According to **Aqib *et al.,* (2019)**, the phenomenon of resistance to antibiotics drugs leads to treatment failure against infections mastitis caused by *S. aureus*, so the vaccine development against mastitis stays an exigent to prevent new infections by *S. aureus* for commercial dairy farms. Anti-*Staphylococcus aureus* vaccines give different results, depending on the type of vaccine, the adjuvant used, and some other factors involved, adding the authors. In humans, levels of mortality caused only by multidrug-resistant bacterial infection in European Union and the Unites States were 25,000 and 63,000 patients per year, respectively. Scientists have warned that the world, in few coming years, will return to a pre-antibiotic epoch plagued by life-threatening microbial infections on the basis of a recent antibiotic resistance gene database that lists the existence of more than 20,000 antibiotic-resistant genes of 400 types

predicted from available genome sequences (**Liu & Pop, 2009**). As a consequence of this new challenge, the discovery of novel antimicrobial agents to which microbes cannot develop resistance easily is one of the major medical concerns of the 21st century (**Das & Patra, 2017**). Finally, it is clear that the resistance phenomenon to antibiotics remains an imminent threat to the effective treatment of bacterial infections, and alternative antibiotic strategies, in this regards, are urgently mandatory. The golden age of antibiotics is coming to an end, and the development of new therapeutic agents to combat infections caused by pathogenic resistant bacteria should be prioritized (**Lewies *et al.*, 2019**).

Nowadays, because of paucity existence of data on the molecular characterization of *S. aureus* in most countries, better understanding of *S. aureus* antibiotic susceptibility profiles and molecular characterization of genes causing resistance are of paramount importance for initiating effective control measures and reducing staphylococcal infections (**Akpaka *et al.*, 2008; Esan *et al.*, 2009**). The use of veterinary drugs remains imperative and play a major role in the control of diseases in cattle populations; a good management and preventive practices in the herds can help the reduction of disease expression and consequently the need to resort to drugs that should be done wisely. Finally, agents must be chosen according to their effectiveness to a type of bacteria which can be evaluated by means of an antimicrobial susceptibility testing. The main objectives of this present thesis were to:

- Investigate the susceptibility of a set of antibiotics representing all groups of the Beta-lactams family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, in years 2003-2004, 2007-2008 and 2017. Moreover, the detection by specific PCR methods of the *blaZ* and *mecA* resistance genes was also evaluated as well as the phylogenetic analysis of partial *blaZ* gene consensus sequences in selected isolates.

- Evaluate the efficacy of a set of antimicrobials from different antibiotics family such as Aminosides family; Tetracyclines family; Macrolides family; Glycopeptides family and Lipopeptides family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, in years 2003-2004, 2007-2008 and 2017. Moreover, the detection by specific PCR methods of the *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *tet(M)*, *tet(K), erm(T), van(A), sal(A), lnu(C), dfrK* and *vga(C)* antibiotic resistance genes was also evaluated in selected isolates.

Literature Synthesis

Chapter I.

What is antimicrobial resistance?

Antimicrobial resistance is a natural phenomenon that causes microorganisms such as bacteria, viruses, parasites and fungi to become insensitive to the effects of antimicrobial drugs, such as antibiotics, previously effective in treating infections. Any use of antimicrobials can lead to the development of resistance. Antimicrobial resistance occurs when microbes (for example, bacteria, viruses, parasites and fungi) evolve in ways that to reduce or eliminate the effectiveness of antimicrobial drugs (for example, antibiotics, antivirals, antifungals and antiparasites) to treat infections by killing or slowing the growth of antimicrobials. When microbes are exposed to antimicrobials, they adapt and become more resistant, increasing antimicrobial resistance in humans, animals, crops and the environment (for example, water, soil) (**WHO, 2014**) (**Figure 1**). In **Figure 1** summarizes causes involved in antimicrobial resistance and impacts of this phenomenon in the near and long terms.

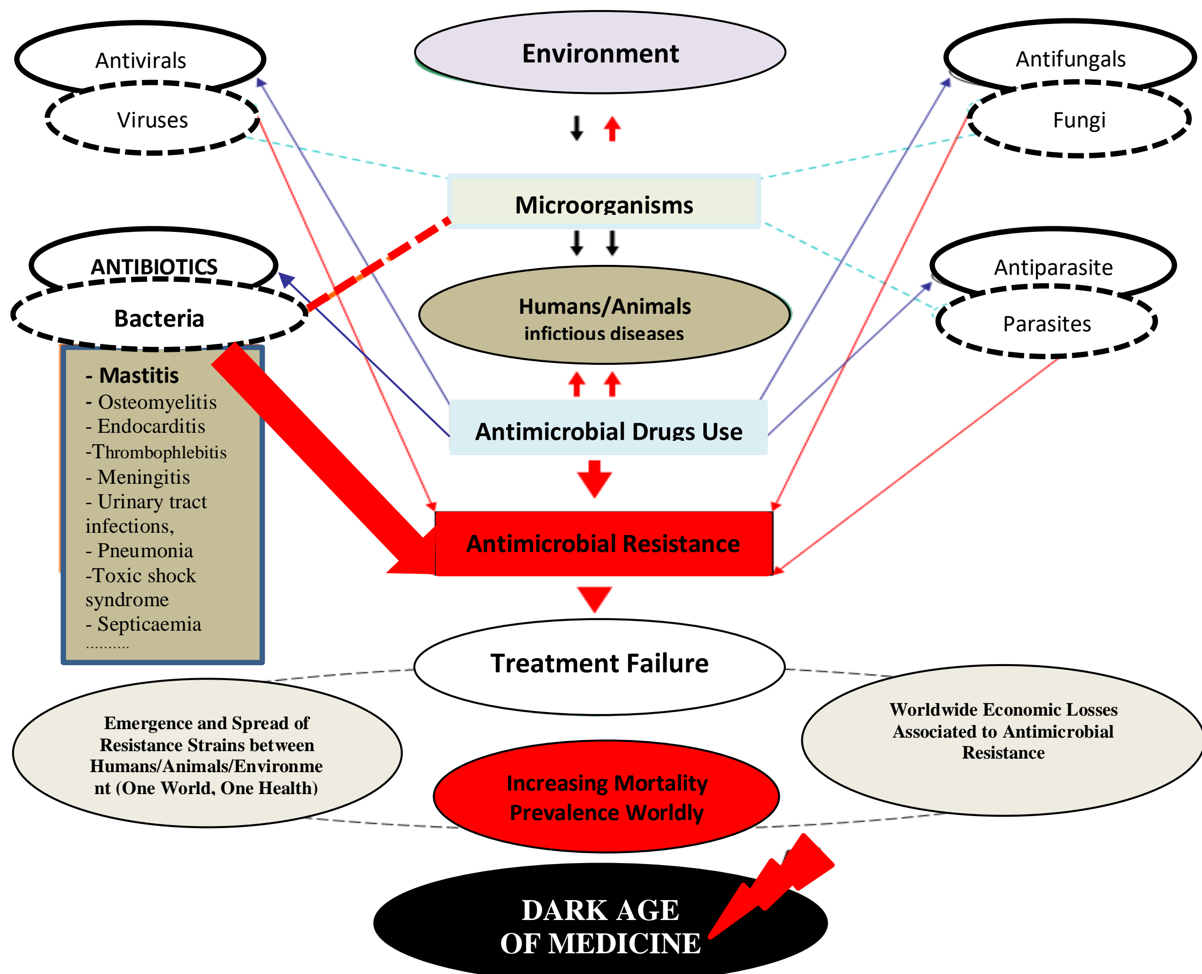

Figure 1: Antimicrobial resistance phenomenon: Causes and impacts.

In **Figure 2** are depicted how:

1) Bacteria cause an infection,

2) Antibiotics are given to kill the bacteria,

3) Some bacteria that cause illness resist the antibiotic treatment,

4) Resistant bacteria continue to multiply and cause infection requiring antibiotics to treat and stop the spread of infection (**PHAC, 2017**).

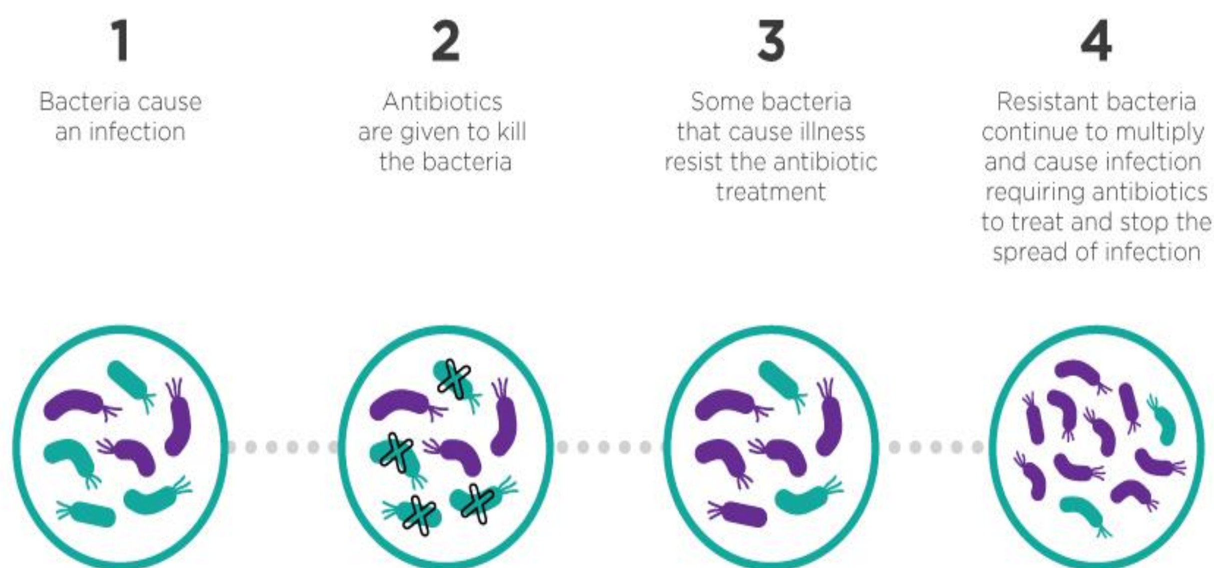

Figure 2: How antimicrobial resistance develops (**PHAC, 2017**).

1. Antimicrobial resistance, a public health problem

1.1. General information and description history of antimicrobials

Before the early 20th century, treatments basis for infections caused by microorganisms was found primarily on **medicinal folklore**. Mixtures with antimicrobial properties that were used in treatments of infections were described over 2000 years ago (**Lindblad, 2008**). Many olden cultures, including the **ancient Egyptians** and **ancient Greeks**, utilized especially selected mold and plant materials and extracts in order to treat infections caused by microorganisms (**Forrest, 1982**; **Wainwright, 1989**). As well, the concept of using of chemicals to alleviate diseases, particularly infectious diseases, dates also not back to the Ancient Egypt only, but also it back to Babylon, the Far East and the Incas civilizations (**Bottcher, 1964**). These early examples of remedies include applying molds to opened cuts and

wounds, and eating radishes, leeks, garlic and onions that are now known to be anti-bacterial (**Kong *et al.*, 2010**).

The make use of antibiotics in modern medicine began with the discovery of synthetic antibiotics derived from dyes (**Goodman & Gilman, 1941**; **Limbird, 2004**; **Calderon & Sabundayo, 2007**; **Bosch & Rosich, 2008**; **Williams, 2009**).

1.2. Etymology of antibiotic

The term 'antibiosis' (which means "against life"), was firstly introduced by a French bacteriologist called **Jean Paul Vuillemin** as a descriptive name of the phenomenon exhibited by these antibacterial drugs (**Foster & Raoult, 1974**; **Calderon & Sabundayo, 2007**; **Saxena, 2015**). In 1877; antibiosis was described, for the first time, in bacteria when **Louis Pasteur** and **Robert Koch** observed that an airborne bacillus could inhibit the growth of **Bacillus anthracis** (**Landsberg, 1949**; **Saxena, 2015**). While in 1942, these drugs were later renamed antibiotics by **Selman Waksman**, an American microbiologist (**Waksman, 1947**; **Calderon & Sabundayo, 2007**; **Saxena, 2015**).

As a result, the use commencement of ‘‘antibiotic term’’ in literature starts since this date with **Selman Waksman** and his collaborators in journal articles to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (**Waksman, 1947**; **Calderon & Sabundayo, 2007**). This definition remains limited because excluded all substances that kill bacteria but that are not produced by micro-organisms (such as hydrogen peroxide and gastric juices). Also this, exclu-ded synthetic antibacterial compounds such as the sulfonamides. However, in recent usage, the term of "antibiotic" is generally applied to any medication that kills bacteria or inhibits their growth, regardless of whether that medication is produced by a microorganism or not (**Scholar & Pratt, 2000**; **Davies & Davies, 2010**). Nowadays, antibiotics are defined as natural molecules with mainly antibacterial activity, originally produced by bacteria or fungi and widely used in medicine for just under a century. The antibacterials can be natural or synthetic and each family has specific properties: the spectrum of activity, the mechanism of action and the bactericidal or bacteriostatic activity arises (**Versluys, 2019**).

There are several families of antibiotics. The main ones are Sulfonamides, Beta-lactamins (penicillins and cephalosporins), Chloromphenicols, Aminoglycosides, Macrolides, Glycopeptides, Glycopeptides, cyclins, Quinolones, Streptogramines, Mutilines, Lipopeptides and Oxazolidinoness. These large families of antibiotics are differentiated by their spectrum of activity, their indications, their route of how they are used, their contraindications and their

adverse effects (**Goodman & Gilman, 1941**; **Limbird, 2004**; **Calderon & Sabundayo, 2007**; **Bosch & Rosich, 2008**; **Williams, 2009; Versluys, 2019**).

Antibiotics remained one of the main innovations of medicine most miraculous discoveries in the 20th century. This discovery has revolutionized the treatment of some dangerous infectious diseases such as pneumonia, tuberculosis and plague, significantly reducing thenumber of deaths related to these diseases (**O'Neill, 2016**). But in recent decades, adaptation mechanisms developed by bacteria have been discovered. These mechanisms have thus allowed them to resist hostile environments, including the presence of antibiotics. These bacteria, which have become resistant, continue to spread on all continents.

Since 1940, the first resistance observation to antibiotics was detected in bacteria against molecules of the sulphonamide family as previously reported by **Ligon, (2004)**. **Figure 3** summarizes the years of introduction to the market of large families of antibiotics.

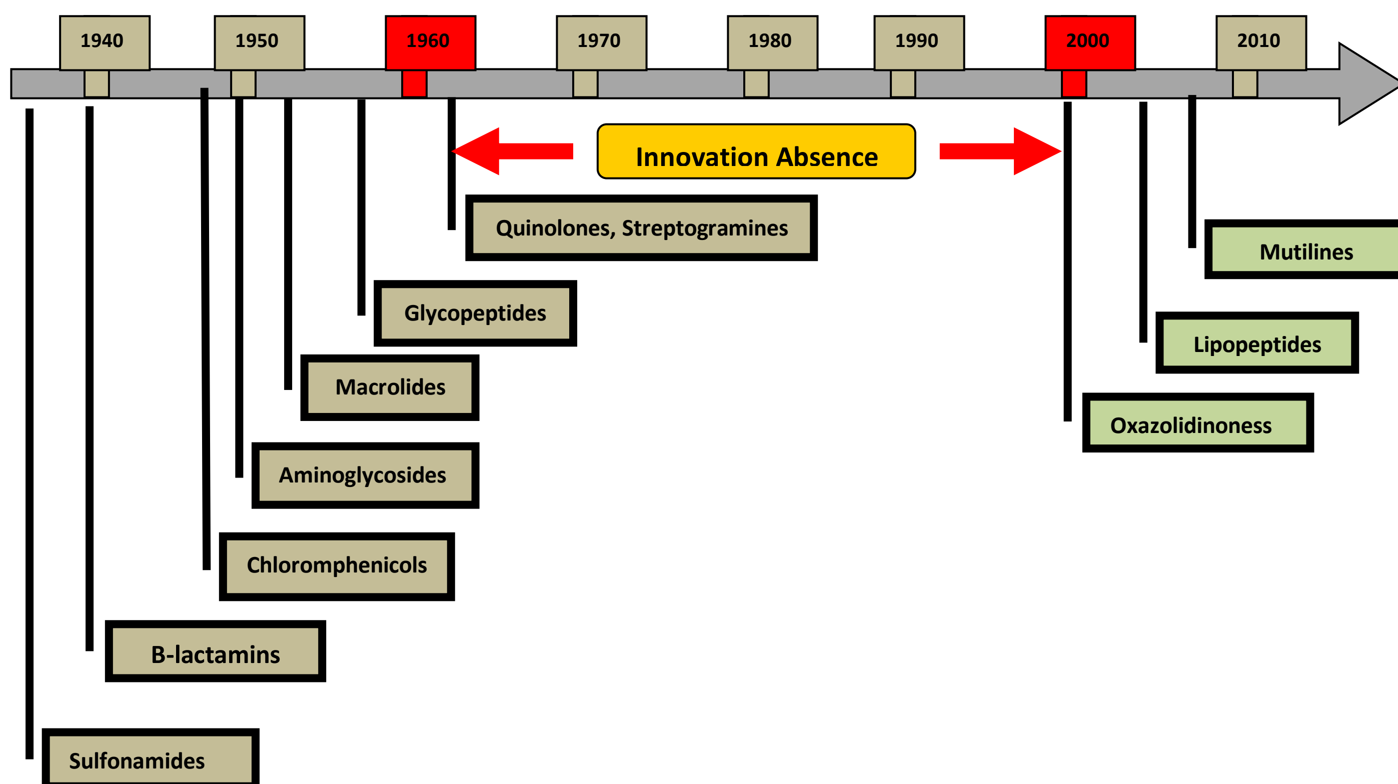

Figure 3: Chronology of the introduction of new classes of antibiotics (**Fischbach & Walsh, 2009**).

1.3. Direct consequences of antimicrobial resistance

Antibiotic resistance is today one of the most serious threats to global health, food security and development. It can affect anyone and any animal, at any age and in any country (**CIS, 2016**). In countries where the antibiotics are dispensed without a prescription for humans or animals, the problem of the emergence and spread of resistance is even more worrying. Similarly, in

countries lacking standardized treatment guidelines, antibiotics are over-prescribed by health workers and veterinarians, and over-consumed by the general public (**OMS, 2018**). Thus, resistance to antibiotic leads to treatment failures, prolonged hospitalization, increased human and veterinary medical expenses, and increased mortality (**OMS, 2018a**).

1.3.1. Reel dangers of antimicrobial resistance in the near and long term

Without effective antimicrobials, our ability to fight infectious diseases will significantly decline. Serious infections will become untreatable, illnesses will become longer and more severe, treatments will become more expensive and toxic, and the risk of death will increase. If infections cannot be prevented or treated, procedures such as organ transplants, cancer chemotherapy and major surgeries (for example caesarean deliveries or hip and knee replacements) may become so risky that they may not be readily available (**WHO, 2014a**).

The Organisation for Economic Co-operation and Development estimates that up to 50.0% of human infections in G7 countries (Canada, France, Germany, Italy, Japan, United Kingdom, and United States) may be resistant to routinely used antibiotics and notes that patients with resistant infections have two to three times higher mortality and risks of complications (**Organisation for Economic Co-Operation and Development, 2015**). The World Bank predicts that by 2050, severe antimicrobial resistance impacts could reduce the global gross domestic product by 3.8% and cause an additional 24 million people to fall into poverty (**World Bank, 2016**) if concerted action is not taken.

Antimicrobial resistance infections have far-reaching implications for human and animal health and potentially the economy. The economic burden of antimicrobial resistance includes impacts on healthcare systems and labour force productivity due to the increased costs of treating patients, longer illness and higher death tolls (**WHO, 2015**). Individuals sick with drug-resistant infections are often not able to work and as a result, they suffer income loss and this contributes to an overall decline in productivity.

1.4. Perspectives in terms of public health

Antibiotic resistance is now reaching dangerously high levels in all regions of the world. Every year in France, 12,500 deaths are for example linked to an infection with an antibiotic-resistant pathogenic bacterium, according to the report of the Interministerial Committee for Health in 2016 (**CIS, 2016**). Worldwide, microbial resistance is currently thought to be responsible for 700,000 deaths per year (**OMS, 2018**).

Alarming estimations issued in the UK Secretary of State for Trade report in 2016 reported 10 million deaths per year by 2050 in the absence of adequate measures (**Figure 4**). Antibiotic resistance could thus become the leading cause of death in the world in the face of cancer (8.2 million deaths), diabetes (1.5 million deaths), diarrhea (1.4 million deaths), and road accidents (1.2 million deaths) (**O'Neill, 2016**).

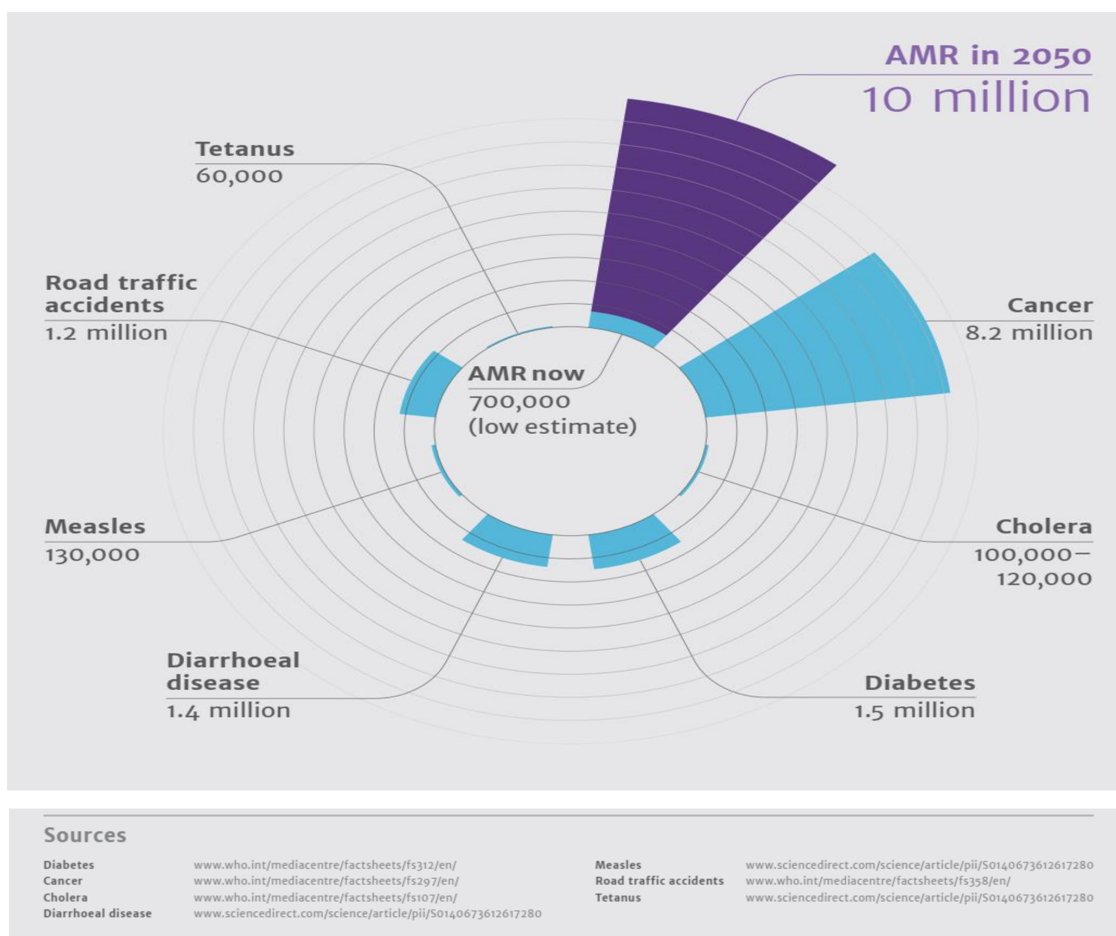

Figure 4: Estimation of the proportion of deaths caused by antibiotic resistance compared to other scourges in horizon 2050 (**O'Neill, 2016**).

More recently; in the Warns UN Report "No Time To Wait" – antimicrobial resistance AMR Could Cause 10 Million Deaths Annually By 2050, **Fletcher, (2019**) reported that deaths from infections resistant to common antibiotics, antivirals and anti-parasitic drugs could increase more than ten-fold to 10 million deaths annually by 2050, warns a ground-breaking United Nations report released today. Antimicrobial resistance is one of the greatest threats we face as a global community. However, several factors also depending on several conditions in each country or even each continent separately will, in one way or another, have a direct or indirect influence on the distribution of the number of deaths due to the phenomenon of antimicrobial

resistance throughout the world. **Figure 5** perspectives death attributable to antimicrobial resistance every year by 2050 in the world.

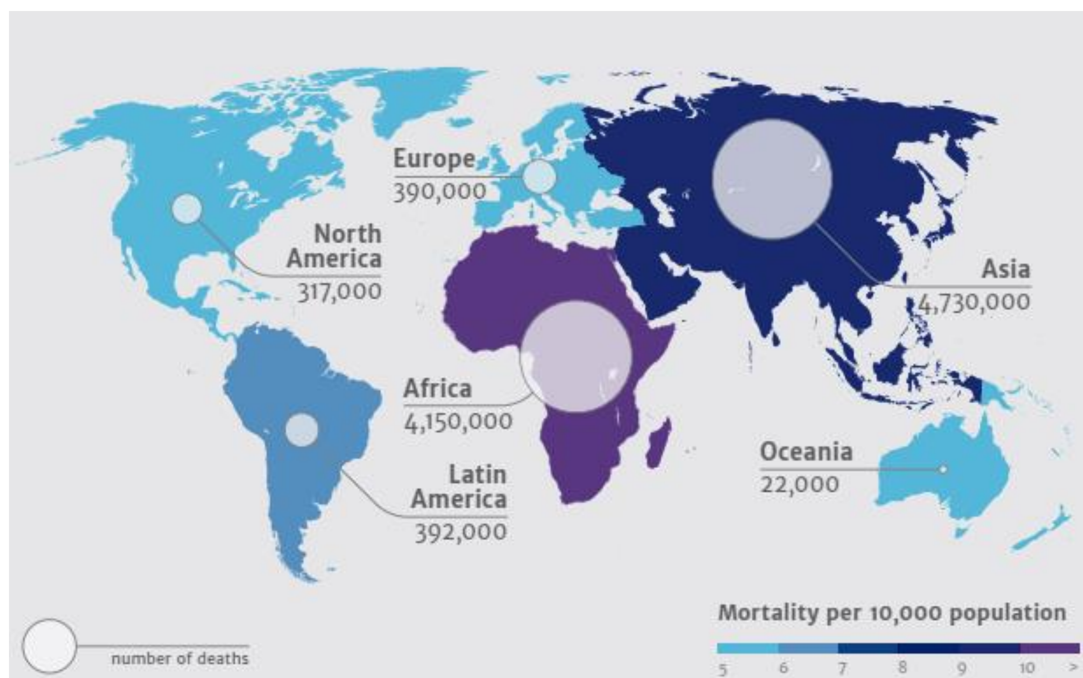

Figure 5: Distribution of deaths attributable to antimicrobial resistance every year by 2050 over continents according to O'Neill, (2014).

It appears from, attributable to antimicrobial resistance every year by 2050 in the continents according to **O'Neill, (2014**), that Asia and Africa would be at the forefront of the death threat continents in few coming decades, they are classified as the two continents that will be most affected in the world by the direct and dangerous effects of antimicrobial resistance by 2050 with about 9 million deaths every year in both continents. After Asia and Africa continents, America (Latin+North) and the European continents will be in the second line of death threat due to the antimicrobial resistance phenomenon by 2050 with more than 700,000 deaths for America and nearly 400,000 deaths every year in Europe. Finally, more than 20,000 Oceanians will die each year because of antimicrobial resistance by 2050. Thus, it seems that the continent of Oceania will be safer from the threat of antibiotic resistance by 2050 compared to the rest of the world.

1.5. The economic cost of drug-resistant infections

According to **O'Neill, (2014**), antimicrobial resistance will have a different impact in different parts of the world. As results of antimicrobial resistance, total World in Gross Domestic Product (GDP) could loss 100.2 trillion of USD by 2050 **(Figure 6**).

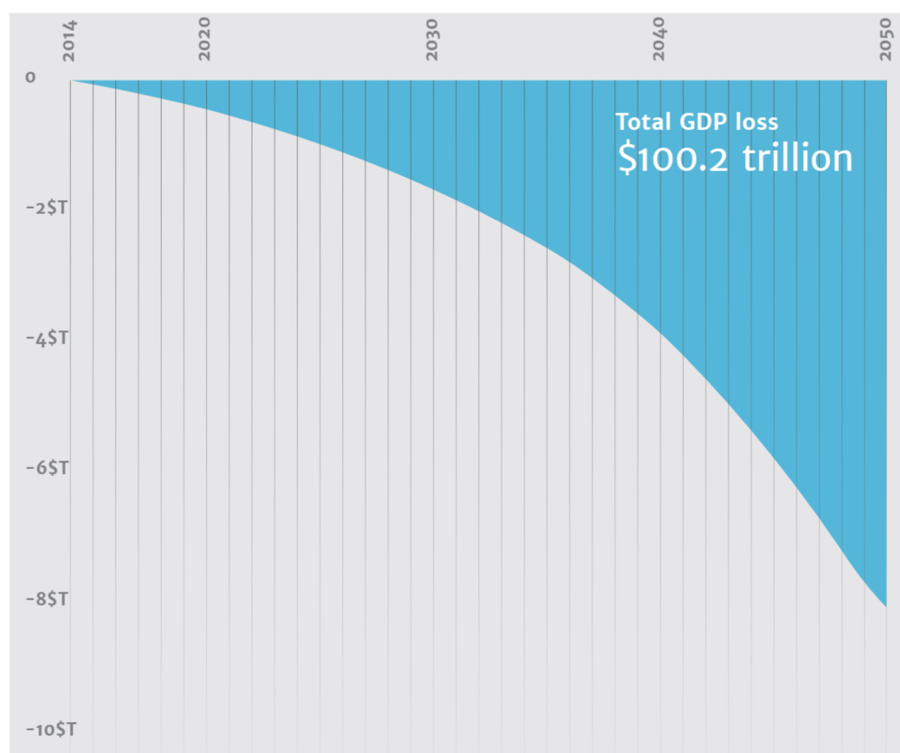

Figure 6: Estimation of the antimicrobial resistance's impact on World in Gross Domestic Product (GDP) in trillions of USD by 2050.

1.5.1. The secondary health effects of antimicrobial resistance: a return to the dark age of medicine?

The real danger posed today by the phenomenon of antimicrobial resistance unfortunately does not associated only at the level of direct infectious disease that could be caused by antibiotic-resistant strains, but this danger will really affect modern medicine in the heart. In medicine, several major disciplines are sensible and are dependant of the success of antibiotic efficacy. Indeed, several actions and interventions in medicine practices and treatment will lead indirectly to appearance of aggravated infections in absence of curative and prophylactic precautions in front, during or after each medical intervention. Thus, the interconnection and complementarily of drugs as a one force to treat several diseases including cancerous diseases, caesarean sections, organ implantation, surgical operations in general will be difficult if not impossible to be realized without the presence of effective and capable antibiotics for eradicating all dangerous infections that threaten human and animal health in each an intervention of a discipline medicine speciality. In this context, **O'Neill, (2014**) reported that despite the staggering size of the figures described. These figures do not capture the full picture of what a world without antimicrobials would look like. One of the greatest worries about antimicrobial resistance is that modern health systems and treatments that rely heavily on antibiotics could be severely undermined. When most surgery is undertaken, patients are given

prophylactic antibiotics to reduce the risk of bacterial infections. In a world where antibiotics do not work, this measure would become largely useless and surgery would become far more dangerous. Many procedures, such as hip operations, which currently allow people to live active lives for longer and may enable them to stay in the workforce, might become too risky to undertake. For example, modern cancer treatments often suppress patients' immune systems, making them more susceptible to infections. Therefore, without effective antibiotics to prevent or treat infection, chemotherapy would become a much riskier proposition.

Despite many medical professionals considering the secondary effects of antimicrobial resistance to be the greatest risk, there remain many unknowns, which have meant that few major studies have looked comprehensively at this impact. According to **O'Neill, (2014**), it is not clear how many more people will get infections when prophylactic antibiotics do not work, nor do we know how many people will opt to take on the risk and still have procedures. Therefore, instead of trying to work out exactly how much the economy would suffer because of these secondary health effects, we have sought to estimate the economic value that these procedures create for society. This gives a sense of what we might stand to lose if antimicrobial resistance rises, with the caveat that we cannot predict how much might actually be lost within this total. We hope that others looking at the impact of antimicrobial resistance will focus more on this area and can build on the initial broad-brush research that we have undertaken.

By way of illustration, we have considered four areas of high-volume medical intervention which have become entirely routine in many parts of the world but are dependent upon the availability of effective antibiotics to make them comparatively low-risk.

We estimate that caesarean sections contribute about 2% to world GDP. Joint replacements add about 0.65%, the vastly improved cancer drugs that have been created since the early 1970s add more than 0.75% and organ transplants add about 0.1%. These are just a small number of the areas in modern medicine that risk being undermined if we do not have effective antibiotics in the future. In aggregate they contribute almost 4% to the world's GDP, worth at least 120 trillion USD between now and 2050. While this total would not be completely lost, when this is combined with the other effects of antimicrobial resistance it shows that the world's economy could lose more than 7% of its GDP by 2050, or a total of 210 trillion USD over the next 30 years. These problems will not just affect high income countries where such surgery is already commonplace, but will also have serious and negative impacts on middle income countries that are expected to build universal health systems over the coming decades (**O'Neill, 2014**).

Rising drug resistance would also have alarming secondary effects in terms of the safety of childbirth, including caesarean sections, with consequential increases in maternal and infant

mortality. The 20th century saw childbirth in high income countries move from being something that carried significant risk to something that we take for granted as being safe: the world witnessed a 50-fold decrease in maternal deaths over the course of that century. Much of this progress could risk being undermined if antimicrobial resistance is allowed to continue rising significantly (**O'Neill, 2014**).

Finally, previous health scares such as SARS have shown that travel and trade can have a much bigger impact on the economy than the health costs assessed by this paper. The reaction is likely to be a growing aversion to travel in a world with dramatic and widespread antimicrobial resistance problems. If there is no effective treatment for malaria, for example, people from malaria-free countries may be unwilling to travel to malarial zones. This should be a major worry for all economies, particularly those reliant on tourism, foreign direct investment or global trade (**O'Neill, 2014**).

1.6. Mechanism of action of antimicrobial agents

According to their principle mechanism of action, antimicrobial agents use for the treatment of bacterial infections can be generally categorized into 4 groups (**Tenover, 2006; Alalem, 2008**):

I. Inhibition of protein synthesis: Bacterial ribosomes differ in structure from eukaryotic cells. Antimicrobial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S ribosomal subunit. Whereas chloramphenicol binds to the 50S subunit of the ribosome (**Figure 7**).

II. Interference with nucleic acid synthesis: Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double strand DNA breaks during DNA replication (**Figure 7**).

III. Interference with cell wall synthesis: Antimicrobial agents that work by inhibiting bacterial cell wall synthesis include the beta-lactams, such as penicillins, cephalosporins, carbapenems, and monobactams, and the glycopeptides, including vancomycin and teicoplanin (**Figure 7**).

IV. Inhibition of a metabolic pathway: Sulfonamides and trimethoprim block the enzymatic pathway for bacterial folate synthesis, which ultimately inhibits DNA synthesis (**Figure 7**).

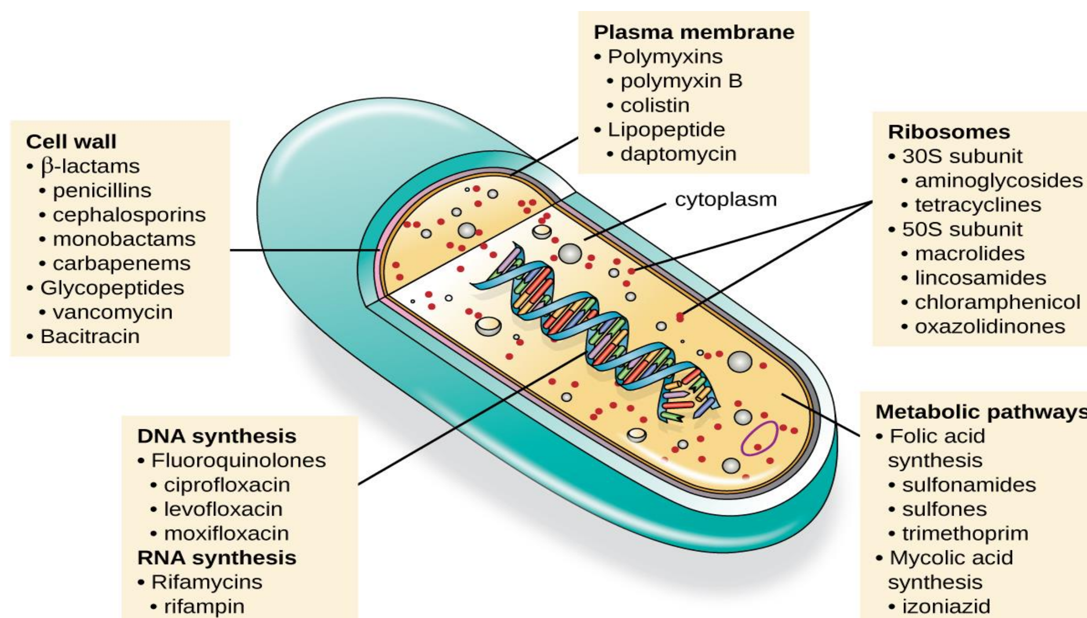

Figure 7: Bacterial targets of antibiotics.

Over the years, resistance has been detected and has spread to the point of posing problems of therapeutic failure. As human and animal health is closely linked, a serious public health problem (**Versluys, 2019**). Antibiotic resistance is a natural defence of bacteria. It may be secondary to the use of antibiotics in a medium or pre-existing in the case of natural resistance. This resistance has a genetic support of natural or acquired origin. Phenotypically, these resistances translate differently: modification of the target of the antibiotic, enzymatic inactivation of the antibiotic and inaccessibility of the targets to antibiotics (**Versluys, 2019**). The use, whether good or bad, of antibiotic products is at the origin of the selection of resistors, but misuse greatly accelerates this phenomenon (**Versluys, 2019**).

1.7. Microbial resistance mechanisms to antimicrobials

As previously described in the work of **Das *et al.,* (2017)**, in the late 1960s when the success of antimicrobial therapies for controlling infectious diseases was at huge, US Surgeon General William H. Stewart made an infamous declaration that “it is time to close the book on infectious diseases and declare the war against pestilence won.” This common sentiment of the medical community at that time has since been proved inaccurate. In the world, the development of resistance phenomenon to common antibiotic compounds in the medical arsenal has brought us to a situation that microbial infections stay the second leading causative of death (**Spelberg *et al.,* 2008**). For this, it appears that understanding the biochemical resistance mechanism has become a momentous biochemical issue. The ability of microbes to resist antimicrobial agents

is dependent on biochemical as well as genetic aspects of the strain (**Das & Patra, 2017**). According to **Dzidic *et al.,* (2008)**, the amazing diversity of antimicrobial resistance mechanisms depends on a set of factors such as microbial strain, nature of the antibiotic, target site, and whether the resistance is conferred by resistance plasmid or chromosomal mutation. On the other hand, the resistance mechanisms are widely distributed in the microbial kingdom and this hinders the efficient antibiotic activity in response to microbial genetic and biochemical flexibility (**Davies & Davies, 2010**). **Figure 8** depicts the resistance mechanism of the microbial kingdom.

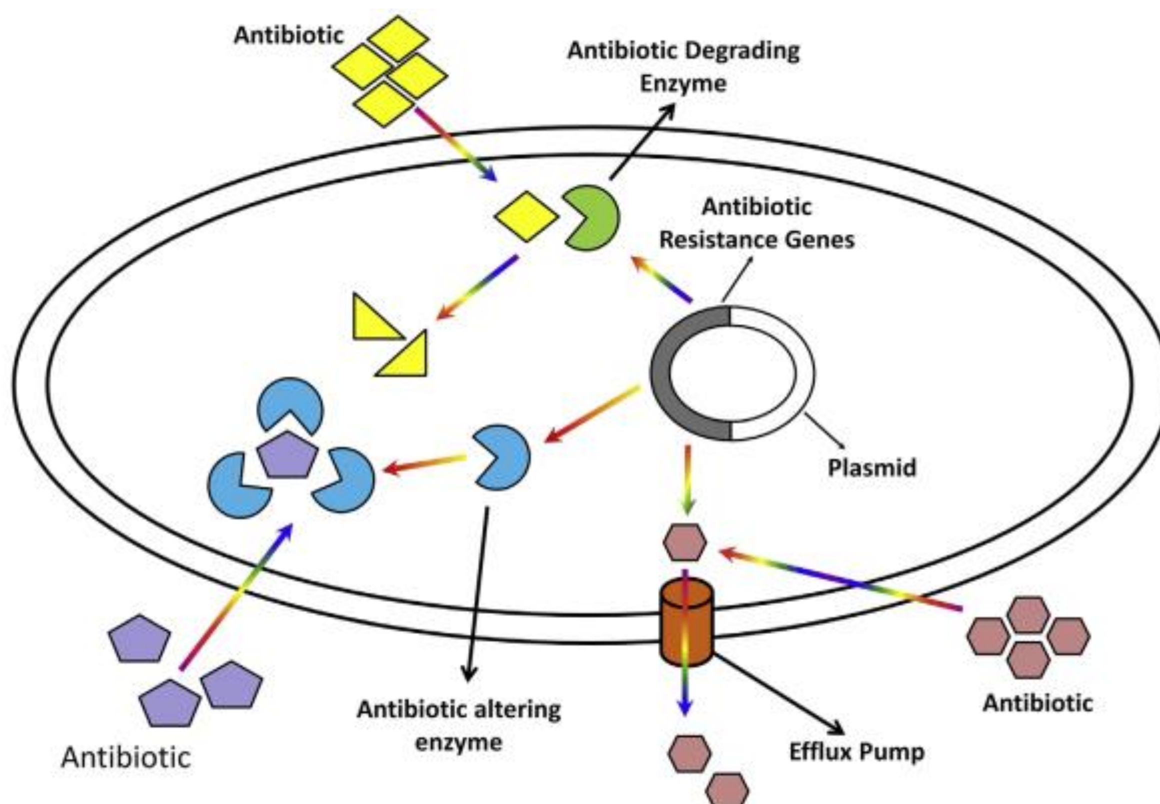

Figure 8: Mechanism of microbial antibiotic resistance (**Byrugaba, 2010**).

1.8. Mechanisms of antibiotic inactivation

Pathogenic bacteria can use several strategies in order to avoid the efficacy of antibiotics. Until now, this most well-known common resistance mechanism involves inactivation of the antibiotic compounds using enzymes that degrade or modify the drug by hydrolysis, group transfer, and redox mechanisms (**Dzidic *et al.,* 2008; Wyk, 2015; Das & Patra, 2017**). The report of **Das** and **Patra, (2017)** explained these mechanisms:

1.8.1. Hydrolysis-based antibiotic inactivation

In the literature reports, several pathogenic resistant bacteria are recognized as microbes capable to produce enzymes that destroy antibiotic drugs by cleaving hydrolytically susceptible bonds as esters and amides in the antibiotic molecule. In this regards, Beta-lactamase is the classical prominent example of these enzymes that cleave the Beta-lactam ring of penicillin and cephalosporins compounds. This action makes the therapeutic used antibiotic lose its activity because it does not possess the ability to bind to PBP to interfere in cell wall synthesis, for example, resistance of Staphylococci and Enterobacteriaceae to penicillin (**Dzidic *et al.,* 2008; Wyk, 2015**). As well, the aminoglycoside group of antibiotics presents other example of antibiotics family that is inactivated by aminoglycoside-modifying enzymes so that the modified antibiotic loses its aptitude to bind ribosome and inhibit protein biosynthesis (**Kapil, 2005; Wyk, 2015**). The resistance conferred to Macrolide antibiotic family is also mediated by the hydrolytic activity of esterases enzymes (**Dzidic *et al.,* 2008**).

According to **Das *et al.,* (2017**), the Beta-lactamase genes are worldwide spread and random mutagenesis of these genes has given increase to extended spectra of resistance. In the literature, it is showed that Beta-Lactamases enzymes can be both plasmid encoded and chromosomal with a wide diversity of around 200 varieties (**Kotra & Mobashery, 1999; Poole, 2004**). Over 180 different extended spectrum Beta-lactamases have been identified that confer resistance to all types of penicillin and third generation cephalosporins, but not carbapenems and cephamycins.

1.8.2. Group transfer-based enzymatic inactivation

Among the important inactivation mechanisms used by pathogenic bacteria, we found the transferase group of enzymes. These enzymes inactivate antibiotics compounds by chemical substitution of the functional group to the periphery of the antibiotic molecule and thus the modified antibiotics are incapable to bind to their target sites. According to **Kumar *et al.,* (2013)**, the modification of the antibiotics is carried out by numerous enzymes for example acetyltransferases (modification of aminoglycosides, chloramphenicol), nucleotidyltransferase (modification of lincomycin, clindamycin, Aminoglycosides), phosphotransferase (modification of Aminoglycosides, Macrolides), glycosyltransferase (modification of macrolides), ribosyltransferase (modification of rifampicin), and thiol transferase (modification of fosfomycin). All these strategies are possible in cell cytoplasm because they require cosubstrate as ATP, acetyl-CoA, NADþ, etc., for activity (**Dzidic *et al.,* 2008**).

1.8.3. Redox process-based antibiotic inactivation

Rarely, antibiotics have been inactivated by oxidation and reduction processes. The best-studied example is oxidation of tetracycline by the enzyme TetX encoded by the TetX gene present in the conjugative transposon of B. fragilis. TetX monohydroxylates tetracycline antibiotics at position 11a, which disrupts the Mg2þ binding site of tetracycline required for antibacterial activity (**Wright, 2005**). Streptomyces virginiae, which produces the type A streptogramin antibiotic virginiamycin M1, protects itself from the antibiotic by reducing the ketone group at position 16 to alcohol (**Wright, 2005; Dzidic *et al.,* 2008**).

1.8.3.1. Target site modification

This manner of antibiotics inactivation is classified as the second most important antibiotic resistance mechanism. In this, the microbial target site of the antibiotic is modified so that antibiotic activity is compromised **(Das & Patra, 2017**). Mutational changes in the site of target reduce antimicrobial susceptibility whereas retaining cellular function (**Spratt, 1994; Dzidic *et al.,* 2008**), for example, peptidoglycan in the bacterial cell wall provides an excellent target for antimicrobial activity. For example, the resistance conferred to family of Beta-lactam antibiotics is obtained by mutation in the penicillin binding domain of PBPs. By this way, bacteria decreases the binding affinity of PBPs for Beta-lactam antibiotics conferring Beta-lactam resistance, for example, resistance to ampicillin encountered in case of *Enterococcus faecium* and in case of resistance to penicillin in *S. pneumoniae* (**Nagai *et al.,* 2002; Kosowska *et al.,* 2004**). Several studies reported that the modification of target sites sometimes requires the microbial cells to undergo other modifications that might compensate for the variation in characteristics of the target sites, for example, *S. aureus* resists activity of methicillin and oxacillin antibiotics by acquisition of a mobile genetic element named *SCCmec* containing *mecA* resistance genes. The *mecA* gene encodes PBP2a, a new penicillin binding protein with high resistance to Beta-lactam antibiotics as compared to normal PBPs. Thus synthesis of cell wall stays uninhibited even under lethal Beta-lactam concentrations, conferring Beta-lactam resistance in *S. aureus* (**Nagai *et al.,* 2002: Kosowska *et al.,* 2004; Dzidic *et al.,* 2008; Wyk, 2015**). The resistance observed to antibiotics that interfere with protein biosynthesis or transcription is obtained by alteration of target site (**Das & Patra, 2017**). In case of macrolide, lincosamide, streptogramin B (MLSB) group of antibiotics, the resistance is conferred by MLSBtype resistance arising from posttranslational modification of the 23SrRNA component of the 50S ribosomal subunit (**Weisblum, 1998**). However, in case of resistance to protein

synthesis inhibitor aminoglycosides, the resistance is conferred by mutation in the 16S rRNA gene. Aminoglycosides producing microorganisms have posttranscriptional methylation of 16S rRNA in the aminoglycoside binding site, which confers microbe aminoglycoside resistance. A matter of serious concern is that emergence of this aminoglycoside resistance mechanism has been reported in nosocomial infections and animal isolates (**Suzuki *et al.,* 1998**).

Flouroquinolone-resistant microbial strains with mutation of bacterial gyrase genes resulting in altered enzymes with decreased fluoroquinolone binding affinity is another classic example of bacterial genetic jugglery (**Hooper, 1999**). Many studies demonstrated that a transmissible mechanism has been reported that relies on the aptitude of aminoglycoside N-acetyltransferases to change a secondary amine on fluoroquinolone leading to reduction in its antibacterial activity (**Nordmann & Poirel, 2005; Depardieu *et al.,* 2007**). Another notable fluoroquinolone resistance that has evolved involves Qnr, which reduces microbial susceptibility to fluoroquinolone by protecting DNA gyrase and topoisomerase IV complex from the inhibitory effect of quinolones. Quinolone exclusion encoded by plasmid-borne oqxAB and qepA genes is a prevalent mechanism leading to quinolone-resistant phenotypes (**Pallecchi *et al.,* 2009**). The rapid emergence of plasmid-mediated quinolone resistance in pathogenic microbes of clinical importance is of high concern, threatening the effectiveness of antimicrobial therapy according to **Das *et al.,* (2017)**.

1.8.3.2. Efflux pump-mediated resistance

Certain microbes use efflux pumps-mediated resistance as an important mechanism for avoiding effectiveness of several antibiotics. Thus, efflux pumps are transport proteins that cause transport of all classes of antibiotics, principally tetracyclines, macrolides, and fluoroquinolones because the antibacterial activity of these drugs is dependent on their intracellular presence. Such pumps are found in Gram-positive as well as Gram-negative bacteria and in eukaryotic organisms (**Das & Patra, 2017**). According to **Nikaido *et al.,* (1999)**, efflux pumps can be specific for a particular class of antibiotic or range of antibiotics of various classes conferring multiple drug resistance. Studies done on bacterial genomic have concluded the intrinsic property of antibiotic resistance in bacteria (**Webber & Piddock, 2003**). Efflux pump genes are part of an operon with expression being controlled by a regulatory gene. Increased expression of these efflux pumps linked with antibiotic resistance is caused by mutation of the regulatory elements controlling efflux pump genes, for example in case of *P. aeruginosa*, mutation of regulator (*mexR*) results in overexpression of *MexAB-OprM* genes encoding the MexAB-OprM efflux pump. This mechanism conducts to augmented resistance

to antibiotics as Beta-lactams antibiotics family (**Gotoh** ***et al.,*** **1995; Köhler** ***et al.,*** **1999; Poole, 2001**). A study by **Webber & Piddock (2003)** reported that microbes with overexpression of the multidrug resistance efflux pump are better equipped to survive antibiotic pressure resulting in further mutation in genes encoding the target site of antibiotics. Synergistic increase in antibiotic resistance with overexpression of efflux pumps and target site mutations in clinical isolates possibly will severely challenge the recent arsenal of antimicrobial treatment in the near future (**Das & Patra, 2017**).

1.8.3.3. Changes in outer membrane permeability

The role of changes in outer membranes permeability, as a mechanism of resistance used by microbes in order to limit the efficacy of antibiotic drugs on these microorganisms, is appeared more important in resistance phenomenon developed by some bacteria. The outer membrane consists of Gram-negative bacteria, which consist of an inner layer of phospholipids and an outer layer containing a lipid A moiety of lipopolysaccharide. This structure of outer membranes allows the transport of small hydrophilic antibiotics including Tetracycline, Beta-lactam, Chloramphenicol, and Fluoroquinolones across the outer membrane by using porin proteins (**Das & Patra, 2017**). In clinical isolates, **Delcour, (2009)** showed the existence of two major porin-based mechanisms conferring resistance to antibiotics. Thus, the first mechanism is reflected by reduction in porins or replacement of one or two major porins by other porins; however, the second mechanism is reflected by presence of specific mutations leading to reduced porin permeability. Resistance to antibiotics caused by a porin-based mechanism has raised major concerns in treating Healthcare-Associated Infections. The raise in minimum inhibitory concentration (MIC) in porin-deficient strains indicates reduction of porin-mediated permeability of antibiotics as a well-organized strategy for the emergence of antibiotic resistance. Among clinical isolates obtained from French hospitals, 44% were revealed to lack porins leading to increased MIC of antibiotics such as cefepime, imipenem, cefotaxime, and moxalactam (**Charrel** ***et al.,*** **1996**). There are several mechanisms that allow pathogenic bacteria to acquire and then transmit resistances phenomenon within bacterial population by using several transmission modalities. For this, we will try to detail and explain these modalities that allow the transfer of resistance mechanisms between pathogenic bacteria.

1.9. Transmission modalities of antimicrobial resistance

1.9.1. Diffusion within a bacterial population

1.9.1.1. Resistance transfers resulting from mutations

In the bacterial genome, chromosomal mutations can occur spontaneously and rarely. If this mutation is at the origin of the acquisition of resistance to an antibiotic, it will be revealed on contact, because of the antibacterial pressure it will exert. If this mutation is viable, it is transmitted to the daughter cells by bacterial reproduction. It is therefore an exclusively hereditary transmission, which often concerns only one antibiotic. This is the case, for example, with *E. coli* whose mutation in the gene coding for the ribosome S12 protein confers them resistance to streptomycin (**Perrot, 1998**; **Guillemot *et al.,* 2006**; **Scott, 2009**).
However, the appearance of mutations is often double-edged for the bacteria concerned. Indeed, they will be an advantage only if the bacteria meet the antibiotic against which they have resistance. On the other hand, these mutants are very often more fragile and less virulent than their original homologues. This would be due to a higher "biological cost" due to an additional synthesis of a priori non-essential proteins in the absence of antimicrobial (**Perrot, 1998**; **Giguère *et al.,* 2007**; **Collectif, 2008**).
This type of transmission, which can therefore be described as vertical and hereditary, represents barely 20% of the resistance encountered clinically (**Maurin, 2013**). Several studies have shown that the appearance of mutations does not depend on the presence or absence of antibiotics. Nevertheless, in the absence of antibiotics, mutants frequently compensate the additional biological cost by other mutations, which are called compensating, which allow them to reduce this burden and remain competitive (**Ferron, 1994; Collectif, 2008; Maurin, 2013**).

1.9.1.2. Transfer of genetic material

There are three types of resistance gene transfer via mobile genetic material transfer **(Figure 9)**:

- **The transformation**, foremost, corresponds to the passive transfer of DNA from one bacterium to another. This type of transfer is partial (less than 1% of the bacterial genome) and therefore limited. It requires a recipient bacteria called "in state of competence" and only the species close to the donor bacteria are capable of doing so. The frequency of appearance of this transfer in the bacterial population is of the order of 10^{-4} to 10^{-6} **(Figure 9)**.
However, if there is transfer, the recipient bacteria acquire new stable and transmissible genetic traits (**Guillemot *et al.,* 2006**). In 1928, Griffith had established that genetic modification could be transmitted from one dead virulent (smooth) bacterial strain to another non-virulent (rough)

strain, so that the rough strain became virulent. McCarty modifies the experiment in 1944, this time incubating the bacteria of the smooth strain with different extracts of cells of the rough strain, containing either the lysed cells, the proteins, or the pure DNA. They subsequently test on Petri dishes the appearance of bacterial colonies having the phenotype of the smooth strain. Despite the fact that experience has shown that transformation only occurs with pure DNA, it was not until the Hershey and Chase experiment in 1952 that the entire scientific community accepted this discovery (**Hershey & Chase, 1952**).

➢ **The transduction** corresponds to the transfer of genetic material from a bacteriophage virus to a recipient bacteria. This recipient can integrate the material and acquire new genes. If the material is recombinant (ability to insert into the genome) and comes from another bacterium, it can acquire genes for antibiotic resistance **(Figure 9)**. The transfer of genetic material can be done directly from a virus to bacteria: it is the conversion. This may also give new characteristics of interest to the bacteria such as, for example, the secretion of diphtheria toxin or the secretion of streptococcus A erythrogenic toxin. The efficacy of this mechanism is slightly better than that of transmission but less than the conjugation. The frequency of occurrence of this phenomenon in the bacterial population approaches 10^{-6} and represents an exchange of approximately 1 to 2% of the bacterial genome (**Guillemot *et al.,* 2006; Maurin, 2013**).

➢ **Conjugation** is an extra-chromosomal mechanism that allows the transfer of a plasmid (mobile and autonomous genetic element present or not in the cytoplasm of bacteria) on which is located a gene called factor F, which has the capacity to encode the biosynthesis of a sexual pili allowing the joining of the two bacteria (donor and recipient) and to mobilize a DNA fragment between the two **(Figure 9)**. If the transferred plasmid is recombinant, it integrates with the chromosome of the recipient bacterium through transposons. Otherwise, it remains free in the cytoplasm and is likely to be, in turn, transmitted to other bacteria (**Guillemot *et al.,* 2006; Maurin, 2013**).

The transfer via plasmids often concerns several families of antibiotics simultaneously and has a high power of dissemination, which makes this mechanism worrying in clinical practice (**Andremont, 2000; Davison *et al.,* 2000; Collectif, 2008**).

Despite a "biological cost" equivalent to that of chromosomal mutations and serving the multiplication of the bacterial strain, the plasmid can transfer virulence genes (in addition to the resistance genes). Thus, the recombinant bacteria are both multiresistant and more virulent, so more "effective". Reversibility is possible because these plasmids can be spontaneously lost by

the bacterium and the number of copies thereof is regulated by bacterial-dependent phenomena (**Smith & Lewin, 1993**). These mechanisms would control a time the dissemination of resistance. Nevertheless, once resistance has emerged and because of its high dispersal power, it is very difficult to get rid of it in a population (**Nelly & Holder, 1999; Guillemot *et al.*, 2006**). This horizontal transmission represents an exchange of 10 to 20% of the bacterial genome and embodies more than 80% of the resistances encountered clinically (**Ferron, 1994; Maurin, 2013**).

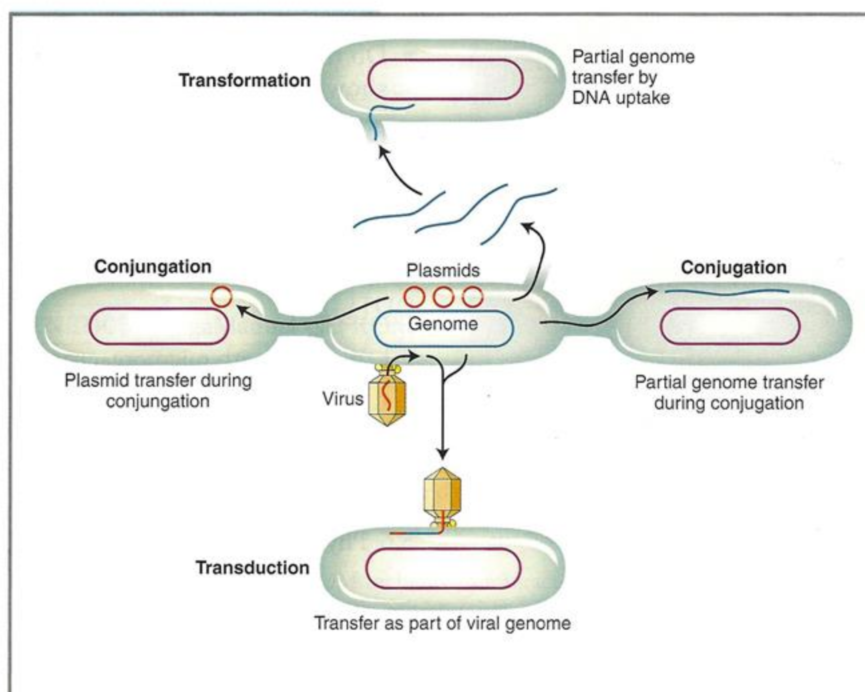

Figure 9: DNA transfer between bacterial cells.

1.10. Antibiotic resistance, a natural mechanism aggravated by several factors

Antibiotic resistance is a natural phenomenon but there existing of several factors that favour it (**CIS, 2016**):

- The misuse of these drugs in humans and animals accelerates the process, as well as its overuse;
- The scarcity of the therapeutic arsenal linked to the industry's disengagement in research and development of new products or the withdrawal from the market of products that are not sufficiently profitable;
- Access to or limited use of diagnostic tools to better target treatment;
- Insufficient application of preventive measures, whether it is a question of controlling the transmission of bacteria or vaccination allowing to avoid the use of antibiotics.

1.10.1. Impact of therapeutic strategies

The use of antibiotics as growth promoters, in prophylaxis, in metaphylaxis and in individual therapy are among the major veterinary therapeutic strategies that can have an effect on the selection and spread of resistance in the bacterial population. Indeed, veterinarians, especially in animal production, use antibiotic therapy and some of their practices can have a significant impact on bacterial resistance. According to French Agency for Food, Environmental and Occupational Health & Safety (ANSES) definition (**ANSES, 2014**), we will try to understand what meaning:

- The use of antibiotics as growth promoters

Growth promoters are defined as "antibiotics used as additives to improve animal growth and performance".

- The use of antibiotics in prophylaxis

Prophylactic treatment is a "treatment applied to healthy animals exposed to a risk factor for infectious disease. Preventive treatment can be individual or collective".

- The use of antibiotics in metaphylaxis

Metaphylaxis is a practice that consists of "treating clinically ill animals and other animals in the same group that are still clinically healthy but whose probability of infection is high because of close contact with sick animals".

- The use of antibiotics in individual therapy

The objective is to treat a single animal for a given infection. The objectives of the treatment are to relieve the animal, limit the spread of the infection to other animals and humans and limit losses in animal production. The treatment put in place is considered by the veterinarian but is often based on a probabilistic thinking and its outcome is not certain.
In the field, clinical examination and anamnesis allow the veterinarian to predict which germs may be at the origin of the clinic but it can sometimes be difficult to properly target the infectious agent. The veterinarian will have to take into account at the same time the scientific constraints, his knowledge and the economic constraints of the breeder. Three criteria are identified to allow treatment to be achieved, with compliance, therapeutic success and the lowest rate of resistance selected (**Sanders, 2005; Bousquet-Mélou *et al.,* 2012; Millemann *et al.,* 2012**):

- **Performing an antibiogram**: To carry out a targeted treatment, the search for the germ involved must be performed. However, it is not always possible in the first intention because performing an antibiogram slows down the implementation of treatment and has a significant cost. Veterinarians and veterinary health professionals agree on the importance of making the most accurate aetiological diagnosis possible in order to use the appropriate antibiotic with as narrow a spectrum of action as possible (**Vandaële, 2012**). Performing an antibiogram is now a mandatory additional examination for the prescription of critical antibiotics.

- **The route of administration**: The local route is the preferred route because it reduces the exposure of commensal flora (which can be seen as a high-risk reservoir) to antibiotics. However, this path is only usable for certain conditions. This is why the veterinarian will have to make a choice depending on the site of infection and the pharmacokinetics of the antibiotic (**Toutain & Bousquet-Mélou, 2012**).

- **The dose and duration of treatment**: Long times and subtherapeutic doses are to be avoided because these two practices do not go in the direction of maintaining a good sensitivity of germs to antibiotics. In the field, to facilitate good compliance with the treatment, one-shot or "long-acting" specialties, chosen according to the time or dose depending on the bacterium / antibiotic pair, can be used to maintain a concentration sufficient for a suitable time with a single intervention of the breeder (**Vandaële, 2012; Bousquet-Mélou *et al.,* 2012**).

1.11. Current status of the menace of antimicrobial resistance

The discovery of the antibiotic penicillin, a fungal metabolite, by Fleming was in 1929, and its subsequent development by Chain and Florey during World War II, that led to the antibiotic revolution. In a few years following the introduction of penicillin, several other antibiotics were described. This miraculous discovery was followed by the development of semisynthetic and synthetic (for example, sulfonamides and fluoroquinolones) antimicrobial agents, which has resulted in an increasingly powerful and effective array of compounds used to treat infectious diseases caused by pathogenic microorganisms **(Giguère, 2013**). But, after each time an antibiotic is introduced in medical practices for treatment of bacterial infections, caused by pathogenic bacteria, in humans or animals the probability of spread of antibiotic resistance looms large (**Austin *et al.,* 1999**). In the literature reports, the pathogenic resistance bacteria, particularly *S. aureus,* have nowadays developed and acquired several mechanisms allowing them to avoid efficacy of antimicrobials. For example, many genes such as *blaZ*, *mec*, *erm*, *aac/aph*, *tet*, *vga* and *van* are among the prevalent resistance genes noted to play a role in *S.*

aureus resistance encoded for several antibiotics such as Beta-lactams, Macrolides, Aminoglycosides, Tetracyclines and Glycopeptides families (**Weisblum, 1995; Vahaboglu *et al.,* 1998; Lina *et al.,* 1999; Martineau *et al.,* 2000; Strommenger *et al.,* 2003; Choi *et al.,* 2003; DeLeo & Chambers, 2009; Fabler *et al.,* 2010; Nizami *et al.,* 2012; Tang *et al.,* 2015; Farid *et al.,* 2015; Pekana & Green, 2018; Ma *et al.,* 2018**). The majority of *S. aureus* strains are now became multidrugs-resistance because of acquisition of several resistance genes encoding for almost of antimicrobials agents.

Nowadays, it is evident that the inappropriate use of antibiotic has conducted to the evolution of pathogenic epidemic-causing organisms into multidrug-resistant forms (**Davies & Davies, 2010**). In developing as in developed countries, despite the high global perils associated with antimicrobial resistance it has been given low priority. The problem is graver in developing countries where use of antibiotics is inappropriate, with easier availability, use of high doses, and relatively cost constraints to replace older antibiotics with new expensive antibiotics augment the chance of increased production of antimicrobial-resistant strains in the majority of the parts around the world (**Kumar *et al.,* 2013**). **Figure 7** summarises and highlights the relationship between antibiotic use and the development of resistance in many target microorganisms.

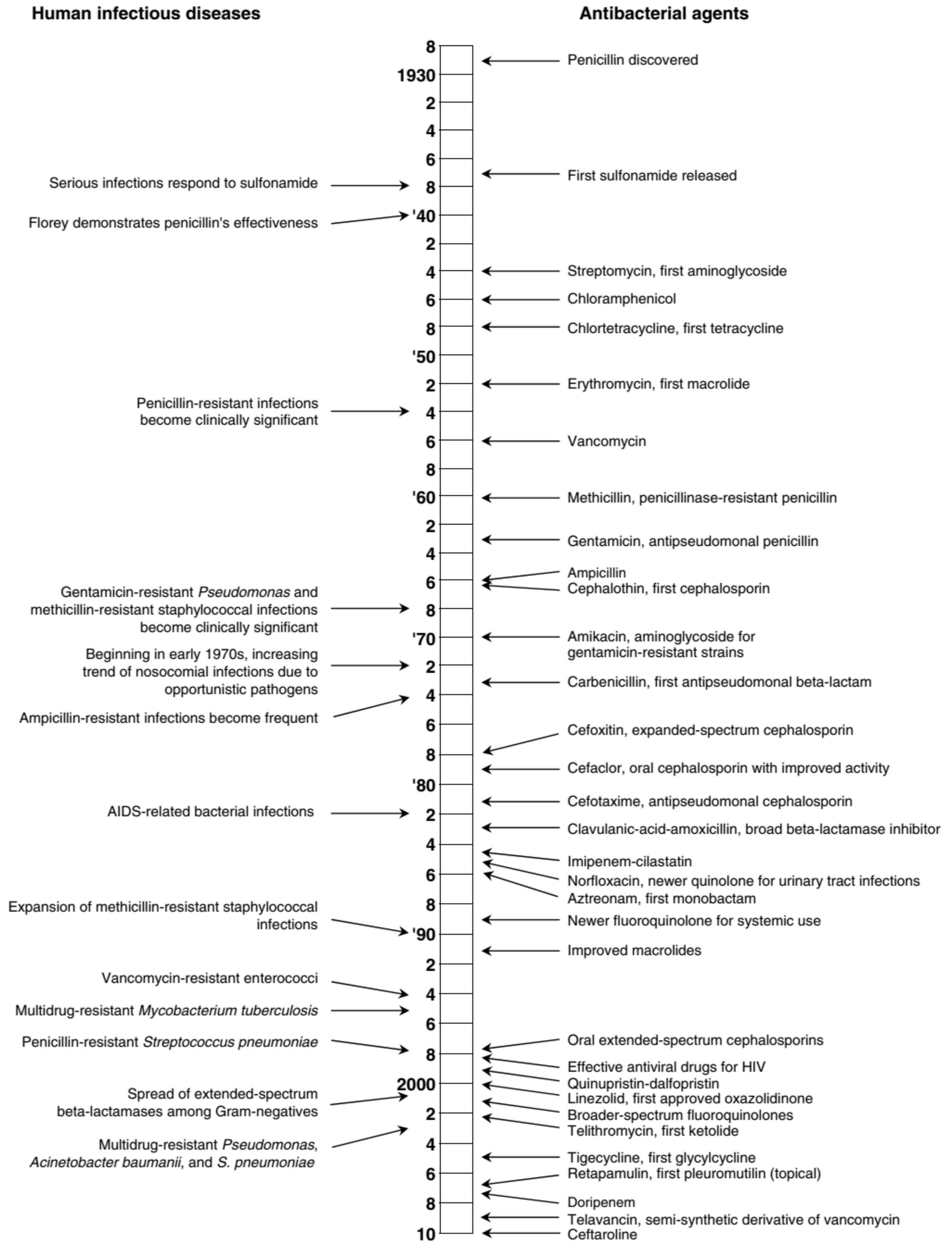

Figure 10: Milestones in human infectious disease and their relationship to development of antibacterial drugs. Modified and reproduced with permission from Kammer, 1982 (**Giguère, 2013**).

The promiscuity between humans and animals are considered as one of the most allowing factor to exchange possibility and spread of resistant pathogenic bacteria between humans and animals on the one hand and the role played by environment in this phenomenon on the one other hand. Thus; in the first time, we will try explain how these pathogenic bacteria can be transmitted from animals to humans and from humans to animals in addition to the role of environment in this regard.

1.12. Exchanges possibility between humans, animals and environment

The spread of pathogens between humans and animals has long been known, but descriptions of the transmission of resistance from animals to humans remain rare (**Andremont, 2000; Madec, 2013**).

The main mode of transmission involves food of animal origin. The most common case concerns the contamination of meat at the slaughterhouse by digestive bacteria. These contaminations, at the origin of Collective Food Toxic Infections (CFTIs), are very often due to human ingestion of *S. aureus*, *Campylobacter* and *Salmonella*. For example, several studies have shown the transfer of resistant *Salmonella* from animals to humans via food (**Teuber, 2001; Madec, 2013; Dweba *et al.,* 2018**). *S. aureus* has been found to be among the most pathogenic and dangerous microbes isolated from different human anatomical sites, livestock and companion animals, foods, food production systems and the environment (**Dweba *et al.,* 2018**). A work by **Hennekinne *et al.,* (2012)** reported that consumption of *S. aureus*-contaminated food caused outbreaks dating back to 1884 where food-borne diseases were found to be caused by the consumption of cheese contaminated with staphylococci. A decade later, a family was discovered to have illnesses caused by the consumption of meat from a cow that had died of fever caused by pyogenic staphylococci **(Hennekinne *et al.,* 2012)**. Other food poisoning outbreaks were reported due to enterotoxin-producing *S. aureus* strains (**Denayer *et al.,* 2017**). According to **Dweba *et al.,* (2018**), these outbreaks highlight the health threats of consuming contaminated foods, highlighting the need to scrutinize food products and production systems for bacterial contamination, specifically *S. aureus*.

The hygienic practices during production of meat play also an important role in the spread of resistance phenomenon if the hygienic degree is not well respected. Because of importance of proteins, high percentage of the population largely depends on beef and pork meat as a protein sources (**Movassagh *et al.,* 2010; Olaoye, 2011**). The work of **Hatakka *et al.,* (2000)** has revealed that the identification of *S. aureus* in meat is a result of improper hygienic practices during handling by the slaughter personnel during meat production. Furthermore, a number of

researchers have showed that infections with antibiotic resistant strains are caused by foods contaminated with antibiotic resistant bacteria (**Nguyen, *et al.* 2005; Pearce & Bolton, 2005; Spanu *et al.,* 2012**) making them an ideal vehicle for transmission of antibiotic resistance (**Pekana & Green, 2018**).

The second route of dissemination of resistance consists of close contact between animals and humans (**Madec & Gay, 2012**). This direct mode of transmission is illustrated by the spread in Denmark of a bacterial clone, MRSA and originally isolated from pigs, to the human population. This spread is responsible for nearly 30% of MRSA cases in human pathology. They are derived from the CC398 MRSA germ and their prevalence is 760 times higher in the hog producer population (**Madec & Gay, 2012**). This example illustrates the power of diffusion in the human population. However, the passage of bacteria from humans to animals is also described. In particular, a case of multi-resistant germ causing mastitis in cattle is reported. The isolation and identification of this germ has revealed a human MRSA of human origin that was carried by the breeder (**Madec & Gay, 2012**). The most worrying strains in the context of resistance transmission between animals and humans ultimately concern mainly zoonotic bacteria (Campylobacter and Salmonella type) and bacteria of commensal flora (*enterobacteriaceae*) **(Toutain, 2007; Kesteman, 2009**).

1.12.1. Transfer and dissemination possibility of MRSA between human and animal

MRSA is regarded as an important pathogen encountered in human medicine, but this strain has also a great capacity to colonize and cause many infections in a range of animal species (**Weese, 2010**). As abovementioned studies, MRSA as well as multi-resistant *S. aureus* strains are reported in veterinary medicine (**Van Duijkeren *et al.,* 2004; Haran, 2012**).

In animal health, many of previous studies have revealed, for example, that MRSA isolates of the clonal lineage ST398 are not limited to pigs only, but can also be isolated from humans, dogs, horses (**Witte, 2007; Denis, 2009)** and from bovine mastitis (**Feßler, 2010**). Due to the low host specificity of MRSA ST398, transfer of such isolates between diverse species of animal, but also between humans and animals, could happen in either direction (**Witte, 2007; Denis, 2009; Feßler, 2010**). In case of animal, **Kadlec *et al.,* (2009)** revealed that during a comparison of the characteristics of the isolates of bovine MRSA ST398 with those previously described for porcine MRSA ST398 isolates showed similarities with regard to the typing results, the virulence patterns and the resistance patterns. Moreover, **Feßler *et al.,* (2010)** indicated that the presence of hospital-related MRSA isolates in either infected dairy cattle or mastitis milk samples can indicate transmission of these isolates between humans and animals.

In general, few studies have dealt with detailed strain characterization; but the majority of these cases, bovine mastitis MRSA isolates associated to those found in human medicine have been lately identified (**Juha´sz-Kaszanyitzky, 2007; Tu¨ rkyılmaz, 2009**).
Furthermore, it appears that detailed epidemiological studies are also obligatory in order to explain the manners by which the dairy cattle have acquired the MRSA ST398 isolates and what role was played by other animals on the farms or farm personnel in the propagation of such isolates (**Feßler *et al.,* (2010**).
According to authors, individuals with close contact with swine, but also with other MRSA ST398-carrying and -shedding animals, remains at risk of being colonized and affected by these isolates of MRSA (**Weese, 2005; Nienhoff, 2009**). People that are colonized by MRSA ST398 can play an important role in the further spread of these strains between different farms and different animal species (**Weese, 2005; Van Duijkeren, 2008**). **Turutoglu *et al.,*** (**2009**) showed that MRSA isolated from bovine mastitis can be originated from human beings, but further studies are needed to investigate the possibility of zoonotic transfer of MRSA, the authors also added.
Certain animals are sometimes infected with MRSA strains originated from human beings, and may either carry these organisms asymptomatically or develop opportunistic infections. The majority of the MRSA encountered in cats and dogs seem to be lineages related with man. Colonization of cats and dogs by these organisms remains frequently transient and tends to occur at low levels; but these strains can be transmitted back to man, and pets might contribute to maintaining MRSA existence within a household or facility. MRSA strains can also by this way be an issue in other settings such as veterinary hospitals, where carriage rates may be higher, particularly during outbreaks in pets, horses and other animals.
In the recent years, MRSA isolates are found only occasionally to be related with bovine mastitis disease (**Feßler *et al.,* 2010**). The first description of MRSA strains isolated from bovine mastitis was 1975 by **Devriese *et al.,* 1975**. Economically, bovine mastitis is the most relevant disease of dairy cattle in which staphylococci play an important role (**Feßler *et al.,* 2010**). A various types of multi-drug pathogenic bacteria are found to be responsible for most cases of bovine mastitis in several countries. But, bacteria of the genus *Staphylococcus* (especially *S. aureus*) are one of the most common multi-drug pathogens that cause mastitis over the world.

1.13. The global response to antimicrobial resistance

Nowadays, no country is immune to the effects of antimicrobial resistance. Drug-resistant organisms that emerge in a single country can quickly spread across national borders due to migration, travel, medical tourism and the global trade of animals and foods. This was demonstrated in 2008 when a Swedish patient travelled to India and became sick with a newly identified multidrug-resistant infection containing the NDM-1 enzyme (New Delhi metallo-beta-lactamase-1) that enables resistance to critically important last resort antibiotics. This resistance has since spread across multiple countries. This case demonstrates how a resistant infection acquired by one individual abroad can easily be brought back to their community and spread across the world **(PHAC, 2017)**.

Given its enormous societal and economic costs, antimicrobial resistance is too great a burden for any one country to bear alone. As such, it must be addressed through a global approach. Antimicrobial resistance requires a coordinated One Health approach across domestic and international boundaries that results in shared solutions for an effective, comprehensive response. A One Health approach acknowledges the interconnection between the health of humans, animals and the environment and the need for collaborative efforts across sectors to improve health for all (**PHAC, 2017**).

The global community is mobilizing through international initiatives to protect human and animal health, conserve antimicrobial medicines and develop innovative responses to mitigate the risk of antimicrobial resistance before the situation worsens. Nations are working together to find ways to share their experience, learn from each other, partner on initiatives and pool resources (**PHAC, 2017**).

In 2015, the WHO Global Action Plan (GAP) on antimicrobial resistance was endorsed at the World Health Assembly by Member States and acknowledged by heads of state and government at the United Nations General Assembly (UNGA) Meeting on antimicrobial resistance in 2016 as the blueprint for action on antimicrobial resistance. As international antimicrobial resistance infection rates continue to rise (**WHO, 2014**), the global community is taking action under the leadership of the WHO, the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO). Both the OIE and FAO have passed resolutions encouraging Member States to combat antimicrobial resistance and to promote prudent use of antimicrobials in animals and agriculture. In line with the GAP on antimicrobial resistance, Codex Alimentarius has recently established an Ad hoc Intergovernmental Task Force on Antimicrobial Resistance to develop guidelines on integrated surveillance and to review and revise the Code of Practice to Minimize and Contain Antimicrobial Resistance to address the entire food chain. International collaboration and

commitments on antimicrobial resistance are also occurring among organizations such as the G7, G20 and the Global Health Security Agenda to strengthen domestic and international capacities (**PHAC, 2017**).

Figure 11 represents how human health, animal health, and the environment are all linked to the issue of antimicrobial resistance. In a continuous circle, you can see how antimicrobial resistance is spread between: 1) humans 2) animals and humans including via food 3) animals 4) the environment, including via contaminated water and fertilizers.

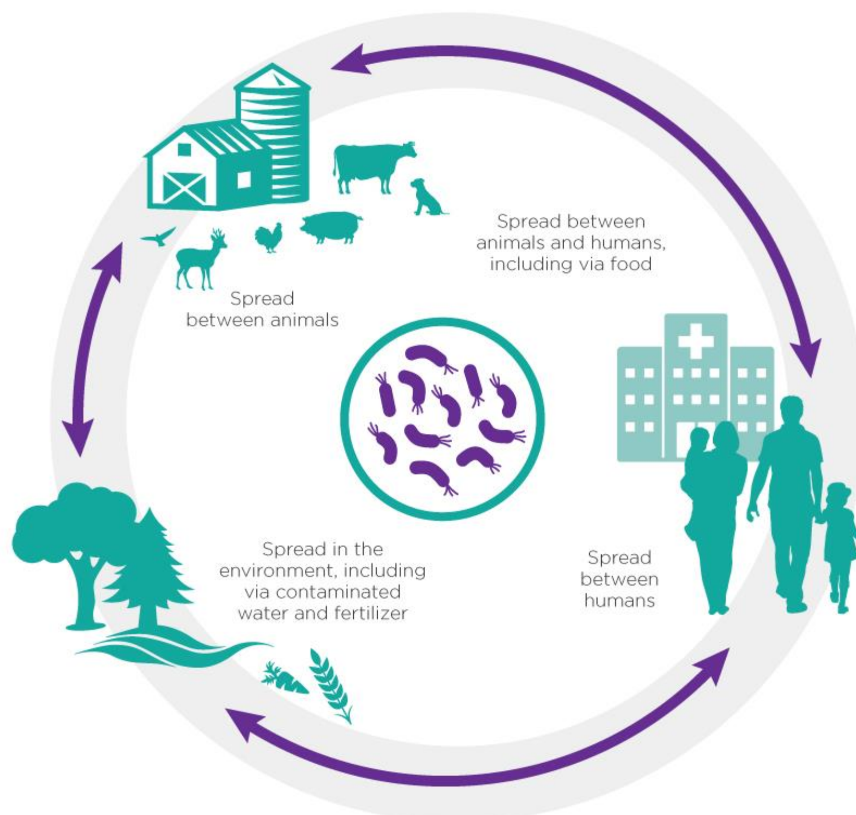

Figure 11: One Health linkages of antimicrobial resistance (**PHAC, 2017**).

1.14. Concept "One World, One Health"

One Health is a collaborative, multisectoral, and trans-disciplinary approach - working at local, regional, national, and global levels - to achieve optimal health and well-being outcomes recognizing the interconnections between people, animals, plants and their shared environment (**One Health Commission, 2019**). Nowadays, antimicrobial resistance is at the heart of the concerns. International scientists and politicians have a good understanding of the challenges and realities of antibiotic resistance. They are therefore maintaining their efforts by developing increasingly demanding programs for the control of antibiotic consumption and the monitoring of resistance.

Globally, a unit in the fight against antimicrobial resistance has emerged between animal and human medicine: the "One World, One Health" approach deployed by WHO in May 2015 hopes to be a springboard for coordinating the global reduction in antibiotic use. Since the 1990s, various initiatives have emerged. National strategies are mainly focused on raising awareness among agricultural sectors and veterinary drug stakeholders (**Versluys, 2019**).
The promiscuity between humans and animals, as well as the current ease of movement, means that the spread of bacteria and/or resistance genes has an impact on all human and veterinary medical activities as well as the environment.
This justifies an intersectoral and inter-ministerial approach, based on the concept of "One World, One Health" (**OIE, 2018**) advocated by the World Health Organization and the World Organisation for Animal Health, in order to control the spread of antibiotic resistance and preserve the immense benefits brought to medicine by antibiotics.
This approach applied to national public policy is necessarily in close coordination with the many international bodies that have made antimicrobial resistance control a public health priority (European Union, G7 and G20, WHO, OIE), which we will focus on later.

1.15. The fight against antimicrobial resistance throughout the world

Antimicrobial resistance is now a major concern. Scientists and national and international policies have clearly understood the challenges and realities of antibiotic resistance. They are therefore maintaining their efforts by developing increasingly demanding programs for the control of antibiotic consumption and the monitoring of resistance.

1.15.1. At the international level

- The World Health Organization (WHO) is a specialized agency of the United Nations (UN) for public health, created in 1948. According to its constitution, its objective is to bring all the world's peoples to the highest possible level of health. It is therefore only interested in the human health consequences of antimicrobial resistance.
- The World Organisation for Animal Health (OIE) is the intergovernmental organisation responsible for improving animal health worldwide, and is therefore the counterpart of the WHO in animal health. It has been in existence since 1924 and covers 180 member countries and territories by 2017.
- The Food and Agriculture Organization of the United Nations (FAOUN) or, more commonly, FAO (Food and Agriculture Organization of the United Nations) is a specialized organization created in 1945 with the ultimate goal of eradicating hunger in the world. However, its missions

concerning food security and activities aimed at making agriculture sustainable makes it an important protagonist in discussions on the theme in the fight against antibiotic resistance.

- The European Commission is one of the main institutions of the European Union (EU) and was established in 1957 with the Treaty of Rome. Several committees such as the Standing Committee on the Food Chain and Animal Health (formerly the Standing Veterinary Committee) depend on it and it is the basis for many EU-wide measures against antimicrobial resistance.

A part from these cornerstone world organizations, each country can have in parallel their own important organizations, agencies and centres specialised in the following and studying the phenomenon of resistance to antibiotics at national level for each country for more effective controlling based on wise good use of antibiotics and traffic control of these drugs in the field. Many other structures also have roles, more or less important, in monitoring resistance, in informing the public (via advertising in particular), in monitoring the application of measures (**Versluys, 2019**).

1.15.2. Global plan of action to combat antimicrobial resistance

Nowadays, antimicrobial resistance threatens the very heart of modern medicine and the long-term viability of an effective global public health response to the constant threat of infectious diseases. Effective antimicrobial drugs are essential conditions for both preventive and curative measures, since they protect patients from life-threatening diseases and ensure that complex procedures, such as surgery and chemotherapy, can be carried out at lower risk. However, the systematic abuse and excessive consumption of these drugs in human medicine and food production has put every nation at risk. Few substitutes are currently under development. If we do not act immediately and in a coordinated manner globally, we will move towards a post-antibiotic era where common infections could be fatal again.

Alarmed by this crisis, the World Health Assembly, held in May 2015, adopted a Global Plan of Action to Combat Antimicrobial Resistance, which sets out 5 objectives (**OMS, 2016**):

1. Increase awareness and understanding of the issue of antimicrobial resistance through effective communication, education and training;
2. Strengthen knowledge and evidence base through monitoring and research;
3. Reduce the incidence of infections through effective sanitation, hygiene and infection control measures;
4. Optimize the use of antimicrobial drugs in human and animal health;

5. Identify the economic case for sustainable investments that take into account the needs of all countries and increase investment in the development of new medicines, diagnostics, vaccines and other interventions.

The Global Action Plan provides the framework for national action plans. It sets out the essential measures that the various stakeholders must gradually take over the next five to 10 years to combat antimicrobial resistance. These measures are structured around the five strategic objectives set out above.

1.16. Alternatives to antibiotic therapy

The ANSES has identified in a report the existence of a large number of products and substances (molecules, plants, plant extracts and micro-organisms) used as alternatives to antibiotics. But it also highlighted the heterogeneity of the data available to assess their safety and efficacy, as well as their ability to select resistant bacteria (**ANSES, 2018**).

The ANSES therefore considers that it is first necessary to initiate a reflection to define, for the main animal sectors concerned, the classes of alternatives that should be the subject of in-depth work as a priority in order to remove uncertainties about their efficacy and safety. Alternatives to antibiotic therapy remain preventive solutions, namely compliance with hygiene rules, proper husbandry, vaccination and nutrition.

The post-antibiotic era has not yet arrived because antibiotics remain the privileged treatment due to their wide distribution, their powerful activity bacteriostatic, or even bactericidal, of their spectrum of activity and the low costs of production in relation to biotherapies.

1.17. Searching for new antibacterial drugs

The improvement of new drugs in the future remained new challenges because of presence of several factors that limited the chance and options of antimicrobial drugs development such as complex regulatory requirements, challenges in drug discovery, and the high cost of drug development coupled with the low rate of return on investment antibiotics provide compared with drugs for the treatment of chronic conditions all contribute to driving pharmaceutical companies out of the antimicrobial drug market. This has left limited treatment options for infections caused especially by methicillin-resistant staphylococci and vancomycin-resistant enterococci. Judicious exploit of the antibiotics at present available and better infection control practices could help prolong the effectiveness of the antimicrobials drugs that are at this time existing. On the other hand, even if we improve these practices, resistant bacteria will continue to develop and new antimicrobial medicines will be needed. The approaches in the search for

novel antibiotics include further development of analogs of existing agents; identifying novel targets based on a biotechnological approach, including use of information obtained from bacterial genome sequencing and gene cloning; screening of natural products from plants and microorganisms from unusual ecological niches other than soil; development of antibacterial peptide molecules derived from phagocytic cells of many species; screening for novel antimicrobials using combinatorial chemical libraries; development of synthetic antibacterial drugs with novel activities, such as oxazolidinones; development of new antibiotic classes that were abandoned early in the antibiotic revolution because there were existing drug classes with similar activities; development of "chimeramycins" by laboratory recombination of genes encoding antibiotics of different classes; and combination of antibacterial drugs with iron-binding chemicals targeting bacterial iron uptake mechanisms.

Today, infectious diseases caused by antibiotic-resistant pathogenic bacteria has been progressively more growing concern in comparison with the last decades. The rapidity with which some bacteria develop resistance considerably outpaces the slow development of new antimicrobial drugs. Since 1980, the number of antimicrobial agents approved for use in people in the United States has for instance fallen steadily (**Figure 12**) (**Giguère, 2013**).

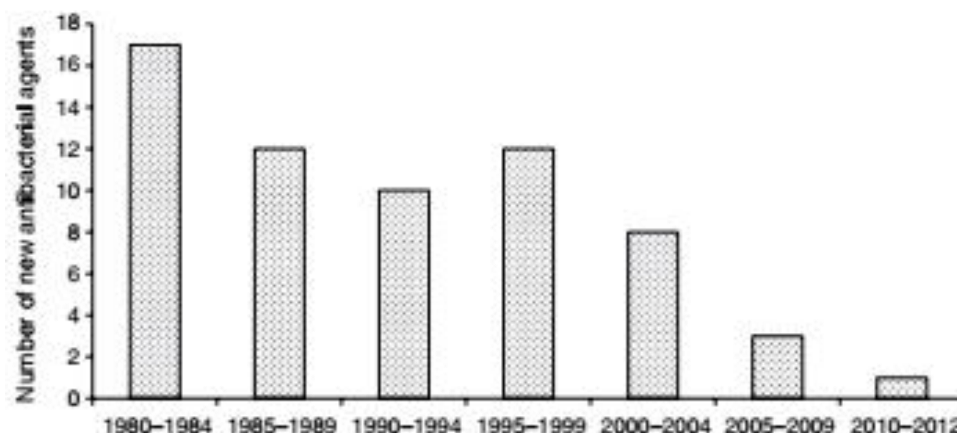

Figure 12: New antimicrobial agents approved for use in people in the United States since 1980.

It is to be noted that the therapeutic employ of these antimicrobial agents in veterinary medicine has usually followed their use in human medicine because of the enormous costs of development (**Giguère, 2013**). The majority (73%) of antimicrobials distributed to animals belong to the same categories as those used in human medicine (**PHAC, 2016**). Nevertheless, a number of antibacterial drugs have been developed specifically for animal health and production (for example, tylosin, tiamulin, tilmicosin, ceftiofur, tulathromycin, gamithromycin, tildipirosin) (**Giguère, 2013**).

Between 1935 and 1968, fourteen new classes of antibiotics were created for human use; but since then, only five of them have been introduced. The need to limit antimicrobial resistance use to maintain the efficacy of antimicrobials makes their development less commercially desirable, and this factor - combined with the long and costly process of commercialization, development and research - means that few new products have entered the market.

1.17.1. Importance of research and innovation approach

Research and innovation are key factors in a multi-faceted approach to overcome the challenges of antimicrobial resistance. They offer tremendous possibilities to improve our understanding of the development and spread of antimicrobial resistance to foster appropriate antimicrobial resistance use to preserve antimicrobial effectiveness, to stimulate the development of new antimicrobials, and to find better diagnostic tools. Research and innovation are also important to support the strengthening of surveillance systems, antimicrobial stewardship and programs.

The complexity of antimicrobial resistance is enormous, and addressing it is beyond the capability and responsibility of any one government, agency or organization. Each country must be prepared to respond to the threat of antimicrobial resistance in order to lessen the health risks to humans and animals in the face of rising rates of drug-resistant infections around the world. Like their international counterparts, governments are employing a One Health approach to tackle antimicrobial resistance. Together with governments, public and private sector partners, including professional associations, industry, academia and the public who have a role to play in antimicrobial resistance must collaborate, coordinate and leverage actions being taken across sectors to minimize duplication and to move in the same direction in an effective and sustained manner.

All around the world, countries are grappling with the lack of new medicines to treat antimicrobial resistance infections. The global community (for example G7 and G20) has made commitments to research and innovation and is seeking effective solutions such as antibiotic development, alternative medicines, vaccines, diagnostics, economic incentives for research and development, and collaboration across countries and sectors. Substantial investments are being made to forge collaborative partnerships to maximize existing and future antimicrobial resistance efforts and to pool financial resources. Today, all countries must consider new approaches to treat resistant infections and examine ways to encourage large drug companies to re-enter the antimicrobial resistance research and development field. Concerning great efforts deployed by some countries in the world, we prefer to present Canada as example in this context for many reasons.

Canada has a strong and collaborative research and innovation culture with expertise in (but not limited to) drug discovery, microbiology, alternatives and adjuvants to antimicrobials, livestock management/housing and vaccine research. Academic institutions, government, non governmental organizations and industry researchers are making important contributions to protect the health of humans and animals against antimicrobial resistance. In academia, the research and innovation landscape is robust and internationally competitive. University-based researchers are working at large teaching hospitals and veterinary schools. Innovation hubs have been established that focus on human health as well as animal and agriculture research and many have strong collaborations with industry. These hubs are applying basic research to advance the development and commercialization of antimicrobial resistance -related products. Canadian researchers are also enlisting commercial livestock producers to develop regionally-appropriate solutions that are acceptable to industry. Complementary research work is being carried out by federal and provincial government researchers to inform antimicrobial resistance research and innovation (**PHAC, 2017**).

Figure 13 shows the groups who play a key role in addressing antimicrobial resistance in Canada. These groups include: the federal government, provincial and territorial governments, academia, human and animal stakeholders, industry, public and health professionals.

Figure 13: An example of groups who play a key role in addressing antimicrobial resistance in Canada.

1.18. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics

The World Health Organization was requested by Member States to develop a global priority pathogens list (global PPL) of antibiotic-resistant bacteria to help in prioritizing the research and development (R&D) of new and effective antibiotic treatments. For this, a coordinating group of eight experts in infectious diseases, clinical microbiology, R&D, public health and infection control were selected to define the protocol. Thus, the prioritization exercise has been performed through the following steps: 1) Selection of antibiotic-resistant bacteria to be prioritized; 2) Selection of criteria for prioritization; 3) Data extraction and synthesis; 4) Scoring of alternatives and weighting of criteria by experts; and 5). Finalization of the ranking of pathogens. Finally, the experts agreed on grouping the pathogens according to the species and the type of resistance and then stratifying the results **in three priority tiers: critical, high and medium** (**WHO, 2017**). *S. aureus* is considered to be one of the most clinically important multidrug-resistant threats globally, according to the recent global priority pathogens list (global PPL) of antibiotic-resistant bacteria by the World Health Organization (**WHO, 2017**). Methicillin-resistant *S. aureus* (MRSA), vancomycin intermediate and resistant *S. aureus* strains are placed second on the list of bacteria of high priority for research and development of new antibiotics (**Tacconelli *et al.,* 2018**) **(Figure 14)**.

WHO PRIORITY PATHOGENS LIST FOR R&D OF NEW ANTIBIOTICS

Priority 1: CRITICAL#

Acinetobacter baumannii, carbapenem-resistant

Pseudomonas aeruginosa, carbapenem-resistant

***Enterobacteriaceae**,** carbapenem-resistant, 3rd generation cephalosporin-resistant

Priority 2: HIGH

Enterococcus faecium, vancomycin-resistant

Staphylococcus aureus, methicillin-resistant, vancomycin intermediate and resistant

Helicobacter pylori, clarithromycin-resistant

***Campylobacter*,** fluoroquinolone-resistant

Salmonella *spp.*, fluoroquinolone-resistant

Neisseria gonorrhoeae, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant

Priority 3: MEDIUM

Streptococcus pneumoniae, penicillin-non-susceptible

Haemophilus influenzae, ampicillin-resistant

Shigella *spp.*, fluoroquinolone-resistant

Mycobacteria (including *Mycobacterium tuberculosis*, the cause of human tuberculosis), was not subjected to review for inclusion in this prioritization exercise as it is already a globally established priority for which innovative new treatments are urgently needed.

* Enterobacteriaceae include: *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., and *Providencia* spp, *Morganella* spp.

Figure 14: Who priority pathogens list for research and development of new antibiotics (**WHO, 2017**).

Chapter II.

What is *Staphylococcus aureus* (*S. aureus*) ?

1. Taxonomy and biology of *S. aureus*

Kingdom:	**Bacteria**
Phylum:	**Firmicutes**
Class:	**Bacilli**
Order:	**Bacillales**
Family:	**Staphylococcaceae**
Genus:	***Staphylococcus***
Species:	***Staphylococcus aureus***

(**NCBI : txid1280, 2019**)

S. aureus is known as one of the famous member of the Staphylococcaceae family, a taxomic group containing 33 other members according to **Freney *et al.*, (1999)** such as *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus* (**Stark, 2013**) while **Holt *et al.*, (1994)** reported that the genus *Staphylococcus* contains 37 species, where 16 of which are encountered in humans. In the genus, the mainly virulent species found for man include *S. aureus* (**Holt *et al.*, 1994**). Staphylococci were before classified in a common genus with *Micrococci* spp. (**Baird-Parker, 1971**) until recently when it was taxonomically placed between the Listeriaceae and Bacilliaceae on the basis of the 16S ribosomal RNA sequencing (**Ahmad *et al.*, 2000; Alalem, 2008**). Subsequently, about 50% of the *S. aureus* genome shares homology with non-pathogenic sporulating *Bacillus subtilis,* signifying that the two micro-organisms are rather close and have evolved from a common ancestor as formerly suggested (**Kuroda *et al.*, 2001**). As different to *Micrococci*, *Staphylococci* contain low guanine/cytosine content and peptidoglycan-bound teichoic-acids in their cell wall structure (**Moreillon, 2005**).

The *S. aureus* have around 2,600 genes and approximately 2.8 Mbp of DNA in its chromosome. Mobile genetic elements, such as plasmids, pathogenicity islands and phages may also constitute part of the species genome (**Barcia-Macay**, **2007; Alalem, 2008**). In *S. aureus*

species, genetic variation is very extensive, with about 22% of the genome comprised of dispensable genetic material.

In general, *S. aureus* is a Gram-positive cell (**Washington *et al.,* 2006**) that it stains Gram positive **(Washington *et al.,* 2006**; **Van Belkum *et al.,* 2009)** and is characterized as non-spore-forming and as facultative anaerobic, non-moving small round shaped or non-motile cocci (**Washington *et al.,* 2006**). It is found in grape-like (staphylo-) clusters. This is why it is called *Staphylococcus*. According to **Howard *et al.,* (1987)**, the species named aureus, refers to the fact that colonies often possess a golden colour when grown on nutrient rich solid media, whilst CoNS form pale, translucent, white colonies (**Figures 15 and 16**).

In the laboratory, *S. aureus* is generally identified by its characteristic Gram-stain, the production of the coagulase enzyme and certain biochemical properties, chiefly the production of catalase and fermentation of mannitol. The coagulase enzyme is considered to be a virulence factor in *S. aureus* that can be cell-bound or free (extracellular) (**Brown, 2005**). A test for coagulase enzyme remains crucial for the differentiation of *S. aureus* from coagulase-negative staphylococci such as *S. epidemidis*, which are common skin commensals.

S. aureus is found to be both a commensal and pathogen of humans and certain animal species (**Feng *et al.,* 2008; van Belkum *et al.,* 2009**).

Figure 15: Group includes CNS whose main representative is *Staphylococcus epidermidis*.

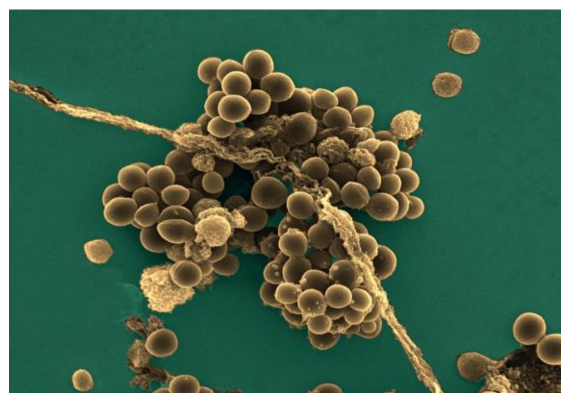

Figure 16: Group includes CPS whose main representative is *Staphylococcus aureus.*

1.1. Discovery and Incidence of *S. aureus*

Micrococci were described by Koch for the first time in 1878 (**Koch, 1878**). Moreover, the identification of *S. aureus* as a human pathogen followed shortly thereafter through the work of Ogston (**Ogston, 1881**). Historically, Alexander Ogston was the first one that could isolate *S. aureus* from the pus of surgical wounds, who initially referred to the bacteria as micrococci in 1881 (**Ogston, 1881**). Their characteristic appearance—grape-like clusters (*staphyle* in

Greek) of sphere-shaped bacteria-prompted Ogston to name the organisms *staphylococci*, which distinguishes them from chain-forming streptococci, also associated with surgical wound infections. The author reported that the injection of staphylococci into the subcutaneous tissue of experimental guinea pigs and mice produced abscess lesions (**Ogston, 1882**). In 1884, Rosenbach succeeded differentiate staphylococci isolated from humans based on the pigmentation of their colonies, proposing the nomenclature *Staphylococcus aureus* and *Staphylococcus albus* for yellow and white colonies, respectively (**Rosenbach, 1884**). The latter species is now renamed *Staphylococcus epidermidis.* Staphyloxanthin, a membrane-bound carotenoid produced by *S. aureus*, is responsible for the yellow pigment that characterized colonies aspect. Pigment production scavenges reactive oxygen species and protects *S. aureus* from phagocytic killing (**Clauditz, 2006**). Soon after their discovery, *S. aureus* isolates were observed to possess an ability to elaborate soluble substances that evoked inflammatory reactions after inoculation into experimental animals (**von Leber, 1888; De Christmas, 1998**).

During the following decades, it became more evident that *S. aureus* ranks among the most common and responsible causes of affected bacterial infections humans, producing a broad spectrum of diseases ranging from superficial skin suppurations to life-threatening septicemias. Along with *E. coli*, *S. aureus* also heads the list of agents that are responsible for hospital acquired infections (**Bhakdi & Tranum-Jenson, 1991**).

In the world, invasive *S. aureus* disease was an important cause of mortality before that antibiotics agent were emerged; patients infected with *S. aureus* frequently died. Mortality rates were as high as 82% for patients with *S. aureus* bacteremia (**Skinner, 1941**).

However, the introduction of penicillin in the 1940s for treating invasive *S. aureus* infections, mortality frequencies decreased drastically to around 25% (**Ladhani, 2004**) and thereby leaded to avoid many deaths from this organism. At that time, penicillin has been described to be the drug of choice used for treatment of infections caused by this organism worldwide; however, resistant *S. aureus* phenomenon were reported as early as 1944. (**Kirby, 1944**) and by end of that decade, approximately 25% of the hospital-associated *S. aureus* strains were resistant to penicillin (**Chambers, 2001**).

In the following years, *S. aureus* strains were resistant to penicillin and then later, to semi-synthetic penicillin derivatives such as methicillin or oxacillin, were developed to treat patients infected with penicillin resistant isolates in 1960s. Unfortunately, just one year after introduction of these new antistaphylococcal penicillins, new resistant strains apeared in the

United Kingdom (**Jevons, 1961**). For the first time, these new strains emerged and became thereafter known under methicillin-resistant *S. aureus* (MRSA) name.

Most recently, methicillin-resistant *S. aureus* (MRSA) strains exhibiting intermediate and complete resistance story to other antimicrobials agents (such as Glycopeptides family, Lipopeptides family, Aminosides family, Tetracycline family, Macrolides family, Fluoroquinolones family, … etc) have been isolated in hospitals and some MRSA strains are now endemic in various community niches. The significant events in modern history of *S. aureus* are given in **Table 1**.

Table 1: The significant events in modern history of *S. aureus* (**Alalem, 2008**).

Date	Event	References
1881	**Ogeston** identifies grape-like clustering bacteria in human pus.	**(Ogeston, 1881)**
1884	**Rosenbach** differentiates staphylococcal species based on pigment.	**(Rosenbach, 1884)**
Pre-1940s	Surgeons fear staphylococcal wound infections; significant mortality from invasive infections observed.	**(Richardson *et al.* 1994; Fluit & Schmitz, 2003)**
1950s	Multi-drug resistant strains of *S. aureus* emerge, resistance spread by phage 80a.	**(Barber, 1961)**
1959	Development of methicillin to treat penicillinresistant *S. aureus.*	**(Richardson *et al.*, 1994)**
1961	**Barber** induces methicillin-resistance in *S. aureus* laboratory strains.	**(Barber M, 1961)**
1963	**Jevons** describes the first naturally occurring methicillin-resistant *S. aureus* (MRSA).	**(Jevons *et al.*, 1963)**
1960s	Resistance to macrolides,tetracyclines, chloramphenicol, amino-glycosides and fluoroquinolones reported.	**(Shanson, 1961; Lyon *et al.*, 1987;)**
Mid-1980s	Genetic basis for methicillin-resistance described; penicillin-binding protein 2a (PBP2a) characterize.	**(Hartman & Tomasz, 1984; Reynolds & Brown, 1985 ; Matsuhashi *et al.*, 1986)**
2002	Glycopeptide intermediate *S. aureus* strains isolated.	**(Anonymous, 2002)**
2000 to present	Increased occurrence of community-acquired *S. aureus* reported among athletic teams and compromised population.	**(Carleton *et al.*, 2004; Palavecino, 2004 ; Kazakova *et al.*, 2005)**

The pathogenesis of *S. aureus* is attributed to the production of an arsenal of several toxins and virulence factors (**Oliveira *et al.*, 2018**).

1.2. Reservoirs and modes of *S. aureus* transmission

1.2.1. The reservoirs of *S. aureus*

In the literature, it is known that *S. aureus* strains are generally introduced or transmitted in hospital settings especially by colonized patients or by hospital staff (**Shanson & McSwiggan, 1980; Ward *et al.,* 1981; Reboli *et al.,* 1990; Bradley *et al.,* 1991; Strausbaugh *et al.,* 1993**). In this regards, colonized patients represent one of the major sources of *S. aureus* in hospitals (**Williams, 1959; Calia *et al.,* 1969; Thompson *et al.,* 1982; Wenzel & Perl, 1995**) and their transfer between different hospitals has conducted in the spreading of certain strains (**Saroglou *et al.,* 1980; Kluytmans *et al.,* 1995**). In colonized individuals, the presence of the same clone of *S. aureus* is often detected in the anterior part of the nose and on the skin, representing an endogenous source of bacteria that can cause infection (**Williams, 1959; Calia *et al.,* 1969; Lidwell *et al.,* 1983; Wenzel & Perl, 1995; Kluytmans *et al.,* 1995**) or allowing their dissemination to other patients (**White, 1961; Ehrenkranz *et al.,* 1964**). About 20 to 40% of healthy humans are estimated to carry *S. aureus* in their nose (**Edmond *et al.,* 1996**) back of the throat and on their skin. As referred, the nose stays the primary niche for *S. aureus* colonisation because each decolonisation of the nose trains in decolonisation of other sites of body (**Wertheim, 2005; van Belkum *et al.,* 2009**). Until now, the role of colonisation at other body sites remains not as well understood (**Wertheim, 2005; van Belkum *et al.,* 2009**).
Members of Hospital staff represent a second reservoir from which *S. aureus* can be transmitted to patients (**Williams *et al.,* 1959**a**; Shanson & McSwiggan, 1980; Ward *et al.,* 1981; Craven *et al.,* 1981; Bartzokas *et al.,* 1984; Nakashima *et al.,* 1984; Coovadia *et al.,* 1989; Reboli *et al.,* 1990; Gaynes *et al.,* 1991; Boyce *et al.,* 1993; Sherertz *et al.,* 1996**). They can be either occasional wearers or permanent wearers. Another potential source of contamination could be staff blouses or work surfaces (**Williams *et al.,* 1959**a**; Shanson & McSwiggan, 1980; Craven *et al.,* 1981; Bartzokas *et al.,* 1984**). Moreover, *S. aureus* has potential to survive for several days on soiled surfaces (**Colbeck, 1960; Beard-Pegler *et al.,* 1988; Farrington *et al.*, 1992**).

1.2.2. Risk factors of *S. aureus* transmission

Factors influencing the risk of *S. aureus* transmission are:
a- The microbiological characteristics of the strains (**Shanson, 1981**). Some strains are more easily transmitted than others, including between different hospitals (**Casewell, 1986; Marples *et al.,* 1986**). MRSA strains that overexpress coagulase or possess multiple copies of the gene

encoding protein A have been found to be more often the cause of epidemics (**Calia *et al.,* 1969; Frenay *et al.,* 1994; Jordens *et al.,* 1989**).

b- The risk factors of the patients (**Shanson, 1981**). The probability of a patient being contaminated with *S. aureus* increases with:

i- Its location in a high-risk unit such as intensive care units, burn units, nurseries;
ii- Surgery intervention;
iii- Prolonged hospitalization;
vi- Presence of catheters or implanted biomaterials (**Thompson *et al.,* 1982; Mylotte *et al.,* 1987**).

c- The policy on the use of antibiotics (**Shanson, 1981**). The use of broad-spectrum antibiotics increases the risk of multidrug-resistant germs (**Daum, 1990; McGowan, 1983; Peterson *et al.,* 1990**).

1.3. Virulence factors of *S. aureus*

In the literature, a variety of virulence factors contribute to the capacity of *S. aureus* to generate infections (**Figure 17**); enzymes (**Table 2**), toxins (**Table 3**), cell-surface proteins, adhesion proteins, factors that aid the *S. aureus* bacteria to evade the innate immune defense, and antibiotic resistance mediate survival of the bacteria and invasion of tissue at the infection site (**Zecconi & Scali, 2013**). Furthermore, some toxins cause specific disease entities.

S. aureus have remarkable ability to cause an enormous range of infections; this function is due, in part, to its aptitude to produce multiple virulence factors as cited above (**Alalem, 2008**). Indeed, *S. aureus* can express proteins to bind fibronectin (**Flock *et al.,* 1987**), fibrinogen (**Boden & Flock, 1989**), collagen (**Patti *et al.,* 1992**), vitronectin (**Liang *et al.,* 1995**), laminin (**Lopes *et al.,* 1985**), thrombospondin (**Herrmann *et al.,* 1991**) and elastin (**Park *et al.,* 1996**) to promote adherence and attachment to endothelial cells and basement membranes. All these proteins are known collectively as MSCRAMMs for microbial-surface components recognizing adhesive matrix molecules. *S. aureus* cells also express Protein A, on its surface, which binds to immunoglobulin G by the Fc region, and is required for full virulence of *S. aureus* (**Ogunniyi *et al.,* 2000**). In general, MSCRAMMs are expressed during exponential growth (exponential phase) and their expression is controlled by the intervention of Agr system while in stationary phase, *S. aureus* produces large numbers of membrane-damaging exotoxins and proteases to promote tissue damage (**Figure 17**).

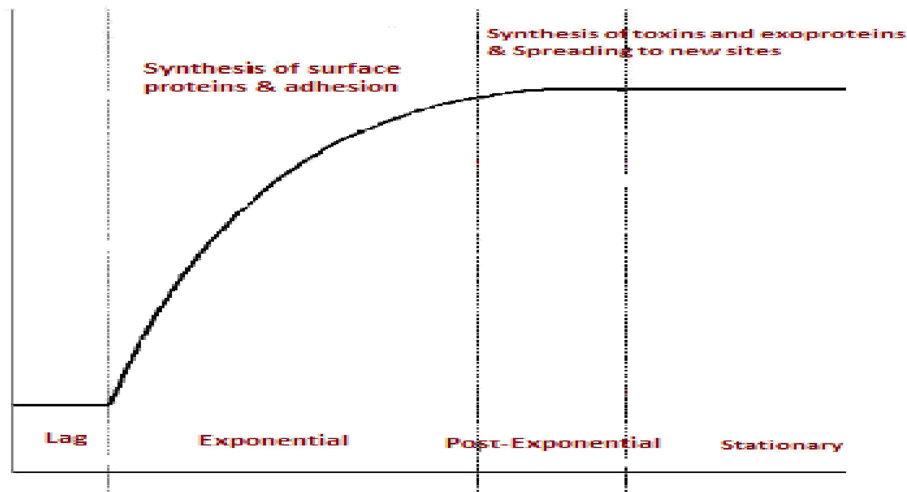

Figure 17: Model of virulence factor production in staphylococcal infections (**Harris *et al.,* 2002**).

In lag phase, bacteria initiate an infection, then enter exponential phase where they multiply and synthesise surface proteins and essential proteins for growth, cell division and adhesion. During post-exponential, crowding activates a density sensing mechanism, resulting in the production of toxins and exoproteins. This enables the bacteria to escape from the localised infection (abscess) during stationary phase and spread to new sites, where the cycle is repeated **(Harris *et al.,* 2002)**.

Invasion of tissues by this organism is especially mediated by production of enzymes such as proteases, nucleases, lipases, staphylokinase and a fibrin-specific thrombolytic (**Colleen, 1998**) (for more information see **table 2** and **3**). Furthermore, certain toxemic strains of *S. aureus* produce superantigens, case of toxic shock syndrome toxin I (TSST-I), to activate large numbers of T cells resulting in proliferation and cytokine release (**Horsburgh *et al.,* 2001**). **Figure 18** shows virulence factors of *S. aureus*.

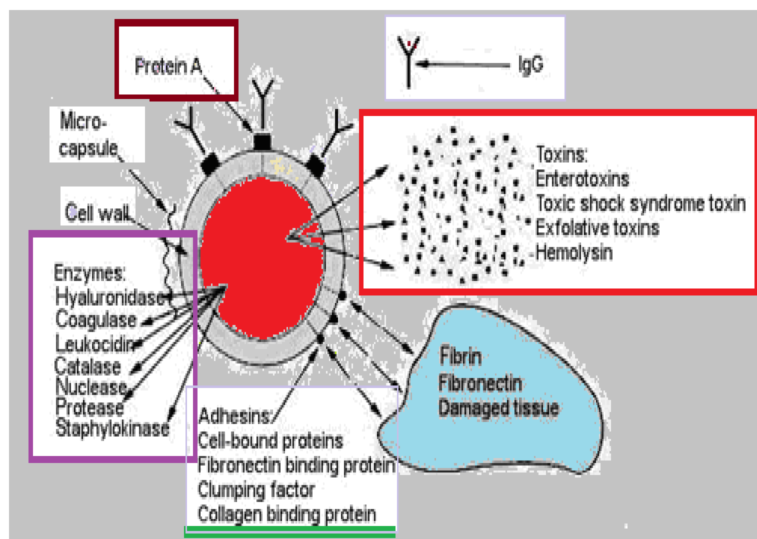

Figure 18: Shows virulence factors of *S. aureus*.

Table 2: Selection of common enzymes regarded as *S. aureus* virulence factors.

Virulence factor	Enzymatic function	Effect as virulence factor in host	Reference
Catalase	- Deactivates free hydrogen peroxide	- Has been shown to be essential for nasal colonization	(**Chavakis *et al.*, 2007; Cosgrove *et al.*, 2007**)
Coagulase	- Binds to protrombin and thereby becomes enzymatically active	- Catalyzes the conversion of fibrinogen to fibrin - Coating the bacteria with fibrin and makes them resistant to opsonization and phagocytosis	(**Kawabata *et al.*, 1986**)
Hyaluronidase	- Degrades hyaluronic acid in connective tissue - Hydrolyzes the intracellular matrix of acid mucopolysaccharides in tissue and, thus may act to spread the organisms to adjacent areas in tissue	- May convert local tissue into nutrients required for bacterial growth	(**Dinges *et al.*, 2000; Washington, 2006**)
Nuclease	- Exonuclease and endonuclease activity	- Contributes to evasion of neutrophil extracellular traps - May degrade host tissue into nutrients required for bacterial growth	(**Dinges *et al.*, 2000; Cheung *et al.*, 2004; Berends *et al.*, 2010**)
Protease	- Degrades human fibronectin, fibrinogen and kininogen - Cleves human α1-protease inhibitor, the heavy chain of all human immunoglobulin classes and elastin	- May contribute to the ability of *S. aureus* to disseminate in host - Aids in tissue invasion	(**Potempa *et al.*, 1986; Prokesova *et al.*, 1992; Massimi *et al.*, 2002; Imamura *et al.*, 2005**)
Staphylokinase	- Plasminogen activator that converts plasminogen to a serine protease, plasmin - More than 67% of *S. aureus* strains express the gene for staphylokinase	- Neutralizes the bactericidal effect by forming complex with α-defensin. - May cleave complement factor C3 - Controls fibrinolysis - The bacteria exploit the proteolytic activity of plasmin to degrade components of ECM as well as fibrinogen for dissemination in the host	(**Rooijakkers *et al.*, 2005; Chavakis *et al.*, 2007; Zecconi & Scali, 2013**)

Table 3: Selection of exotoxins regarded as virulence factors of *S. aureus*.

Virulence	Factor	Function Virulence effect on host	References
Exfoliative toxins	- Glutamate-specific serine proteases that digest desmoglein 1, a keratinocyte cell-cell adhesion molecule. - Exfoliative toxins (ETs) act as "molecular scissors" facilitating bacterial skin invasion - Prevalence of *eta* and/or *etb* range from 0.5-3% in MSSA but 10 % of MRSA strains have been found to be *eta* positive	- The ETA and ETB are the two most important isoforms and they are associated with staphylococcal bullous impertigo and staphylococcal scalded skin syndrome - ETA ETB ETC (not associated with human disease) and ETD Mediate superantigen activity	(**Becker *et al.*, 2003; Kato *et al.*, 2011; Peacock *et al.*, 2002; Sila *et al.*, 2009; Zecconi & Scali, 2013**)
Hemolysins	- Pore forming toxin with cytolytic effect onerythrocytes and monocytes (α–toxin) - Cytolytic activity on cytokine containing cells(β –hemolysin also known assphingomyelinase C) Neutrophil and monocyte binding (δ –hemolysin)	- The vast majority of the hemolysins are haemolytic - α-toxin has pro-inflammatory properties on host	(**Chavakis *et al.*, 2007; Zecconi & Scali, 2013**)
Leukocidines	- A bi-component pore-forming leukotoxin. Consists of one class S protein and one class F protein. The subunits form a ring with a central pore, through which cell contents leak - Different members of the group are γ- hemolysin (hlg), Panton-Valentine leukocidin (PVL) and Leukocidins D, E, M (LukD, LukE, LukM)	- Kills leukocytes - PVL stimulates and lyses neutrophils and macrophages - γ-toxin is haemolytic	(**Chavakis *et al.*, 2007; Grumann *et al.*, 2013; Kaneko & Kamio, 2004**)
Staphylococcal Enterotoxins	- Gastroenteric toxicity; immunomodulation via superantigen activity	- Causes food poisoning - A tleast 20 serologically different staphy-lococcal superantigens have been described, including SEs A to V	(**Chavakis *et al.*, 2007; Pinchuk *et al.*, 2010; Zecconi & Scali, 2013**)
Toxic shock Syndrome toxin	- Toxic for endothelium, direct and cytokinemediatedMediate superantigen activity	- The toxin causes the rare condition 'toxic shock syndrome'(TSS) - These infections are characterized by a rapid onsetwith high fever, rash, vomiting, diarrhea and multiorgan failure	(**Chavakis *et al.*, 2007; Peacock *et al.*, 2002; Zecconi & Scali, 2013**)

1.4. *S. aureus* biofilms factor and chronic infection

The formation of biofilm phenomenon is regarded as an important contributing factor for the establishment of chronic infection caused by the opportunistic pathogen *S. aureus* (**Kiedrowski & Horswill, 2011**). *S. aureus* is easily able to form biofilms on host surfaces such as it is bone (**Brady *et al.*, 2008**), cartilage, and heart valves (**Parsek & Singh, 2003**), as well as on strange body implants, including orthopedic devices and catheters (**Costerton, 2005**).

The mature biofilm is characterized by a composed of a community of cells encased in an extracellular matrix. This composed structure furnishes original resistance to the innate immune system and other antimicrobials and by this way promotes bacterial persistence (**Patel, 2005; del Pozo & Patel, 2007**). Various factors contribute to biofilm recalcitrance. Firstly, the extracellular matrix structure provides a protective barrier against antimicrobials, reducing thereby their permeability into the biofilm. The immune system components such as macrophages (**Thurlow *et al.,* 2011**) and large molecule immunoglobulins and superoxides possess limited aptitude to invade biofilms. Conversely, antibacterial agent with smaller molecules may liberally transverse the matrix material and does not always provoke bacterial killing; therefore, the importance of this mechanism remain not fully understood (**Stewart *et al.,* 2002**). The metabolic state of bacteria that reside into the biofilm and their profiles of gene expression are regarded as other contributors. Largely these are dormant, nongrowing cells that display gene expression patterns similar to that of stationary phase cultures (**Costerton, 2005; del Pozo & Patel, 2007**). Whilst, this state encourages antibiotic resistance, the variations in gene expression may also actively modulate host immune functions by attenuating proinflammatory responses (**Thurlow *et al.,* 2011**). As well, propagation of antibiotic resistance mechanisms by means of gene transfer take place more frequently within biofilms and biofilm conditions select for mutants with development properties of enhanced biofilm. This is particularly clear in the frequency of spontaneous agr mutants in biofilms (**Yarwood *et al.,* 2011**). **Figure 19** shows different stages development of *S. aureus* biofilm.

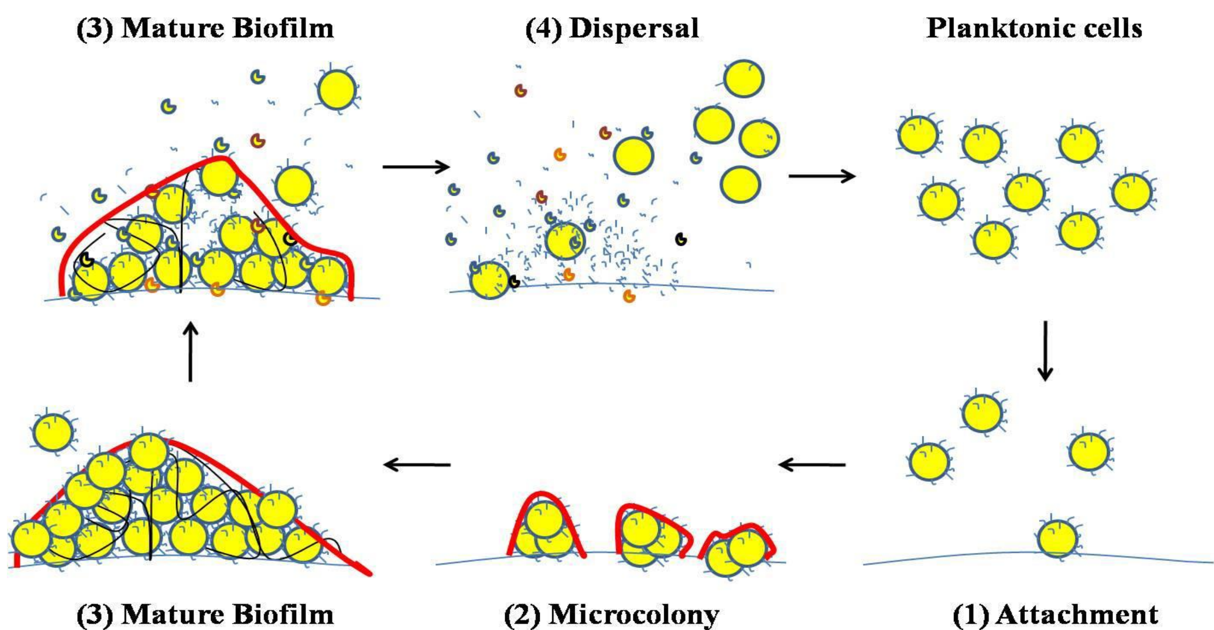

Figure 19: The stages development of *S. aureus* biofilm (**Mootz, 2013**).

Free-floating *S. aureus* cells exhibit qualities seen during planktonic growth. This development passes normally through four essential stages:

1- The first stage is always initiated by an initial attachment of individual cells to an abiotic or biotic surface via their MSCRAMMS.

2- The second stage is characterized by Cells grow and divide, eventually developing microcolonies that contain biofilm matrix material.

3- The third stage is known by an extensive production of matrix material leads to the establishment of a mature biofilm. These structures of biofilm, in this state, are characterized by their enhanced resistance to antimicrobials agents. In addition, biofilm cells are dormant and differentiated from planktonic cells by variations in their profiles of gene expression. Under certain conditions, subsets of cells will detach from the biofilm by means of low level production of matrix altering enzymes.

4- The last stage of development results in the appearance of erosion, dispersion and autogenous detachment under environmental conditions that can alter the regulatory scheme of the bacterium resulting in high level exoprotein production which conducts thereby to destruction of the biofilm matrix and dispersal of cells from the biofilm. Finally, these cells revert back to a free floating planktonic growth state, can also colonize new surfaces thus completing the cycle and could be again susceptible to antimicrobials (**Mootz, 2013**).

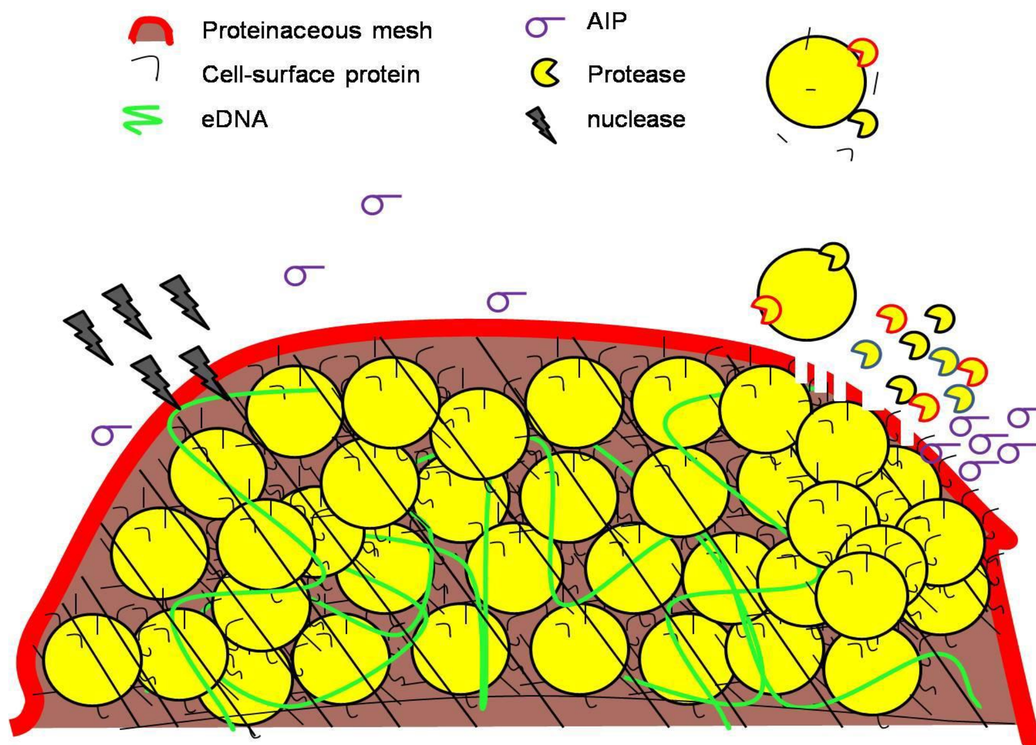

Figure 20: *S. aureus* USA300 extracellular matrix composition (**Mootz, 2013**).

According to **Mootz (2013**); in CA-MRSA, protein and eDNA remains the biofilm matrix principal constituents. Protein components essentially consist of surface-associated proteins such as MSCRAMMS and membrane-spanning proteins, as well as secreted proteins. Furthermore, proteins of cytoplasm have also been found in the biofilm matrix structure. These proteins could be remnants obtained from the lysis of cells that occurs for the period of biofilm initiation and are essential for eDNA deposition and biofilm formation. The importance of various biofilm matrix components has in large part been identified through the utilization of exo-enzymes, such as nuclease and proteases, to disturb mature biofilms. These enzymes could also participate in the natural development stages of biofilms as agr-mediated biofilm dispersal is due to overexpression of secreted proteases.

In general, the mechanism by which biofilms generated by certain bacteria resist antimicrobials appears to be multifactorial. Due to biofilm recalcitrance, treatment options all limited and typically involve removing infected devices or tissues according to **Darouiche, (2004)**.

These strategies are not ideal for patient care and further research is needed to understand the molecular mechanisms of *S. aureus* biofilm development to improve therapeutic strategies for treating chronic infections.

1.5. Action of *S. aureus* in case of sever disease

In the case of severe disease caused by *S. aureus*, the infection can not be only explained by the action of a sole virulence factor, but and it is likely that a set number of various factors operating collectively in the pathogenic process. This hypothesis is supported by studies performed in animal models where the infection caused by a mutant isolate, deficient in a single virulence determinant, is compared with the infection caused by the wild type strain. According to authors, these studies have indicated a decrease in severity of the infection (**Moreillon *et al.,* 1995; Hienz *et al.,* 1996**).

The *S. aureus* survival in the host remains important for pathogenesis. The bacteria can be protected by an intervention of polysaccharide capsule that inhibits opsonization by complement and by this way escapes phagocytosis according to **O'Riordan & Lee,** (**2004**). It may also secrete cytolytic toxins and tissue-cleaving enzymes according to **Dinges et *al.,* (2000)**. Furthermore, *S. aureus* can express a large number of factors of adhesion that play an important role in mediating interactions with host cells and extracellular matrix (ECM), allowing thereby a *S. aureus* efficient colonization as previously described by **Foster, (1998)** and by **Chavakis et *al.,* (2005)**.

S. aureus is among the types of bacteria that have developed multitude strategies against the antimicrobial peptides, the complement system, and the recruitment and actions of phagocytes (**Chavakis *et al.,* 2007**) all of which are strategies against the innate immune response of the host (**Foster, 2005**; **Rooijakkers *et al.,* 2005**).

1.6. Methicillin-Resistant *S. aureus* (MRSA)

S. aureus is regarded as an important opportunistic pathogen frequently carried asymptomatically on the human body. In the world, antimicrobials resistant phenomenon generated by bacteria of *S. aureus* is a mounting problem among infected patients.

In human medicine, antibiotic resistance is mainly related with increases in healthcare costs, length of hospital stay, and patient morbidity and mortality (**Treakle, 2009**). Methicillin (oxacillin)-resistant *S. aureus* (MRSA) strains stay between the large threatening bacteria that implicated in nosocomial infections (**Unal *et al.,* 1992; Vannuffel *et al.,* 1998; McBryde *et al.*, 2004**). Methicillin is Beta-lactam antibiotic invented in order to treat infections caused by penicillin-resistant *S. aureus*; but, MRSA was observed 2 years soon after the antibiotic was introduced in 1961 (**DeLeo & Chambers, 2009; Simonetti *et al.,* 2011; Kejela & Bacha, 2013**). It is estimated that 1.5% of the population (~4.1 million persons) is colonized with MRSA leading to at least 94,000 invasive infections and over 18,000 deaths annually in the United States only (**Frana *et al.,* 2013).**

Worldwide, Methicillin-Resistant *S. aureus* (MRSA) is a scientific expression attributed to each *S. aureus* strain that have acquired a gene that makes it resistant to a large group of antibiotics called the Beta-lactams, which include penicillins and cephalosporins **(Vorgelegt von, 2011; CFSPH, 2016)**. It has evolved an aptitude to survive treatment with beta-lactamase resistance Beta-lactam antibiotics including methicillin, dicloxacillin, and oxacillin **(Vorgelegt von, 2011)**. In general, strain of *S. aureus* that is resistant to methicillin, oxacillin, nafcillin, cephalosporins, imipenem, and other beta-lactam antibiotics (**Maryland, 2001**).

Resistance of MRSA against Beta-lactam antimicrobials is normally conferred by a gene called *mec*A that encodes the production of an altered penicillin-binding protein (PBP2a) **(Turutoglu, 2009**; **Kreausukon, 2011)**. The *mecA* gene is principally (harboured) localized on a mobile genetic element named the Staphylococcal Cassette Chromosome *mec* (SCC *mec*) and has very high levels of homology in MRSA (**Beck et *al.,* 1986; Bignardi *et al.,* 1996; Fluit *et al.*, 2001**). For that reason, Beta-lactam antibiotics are not effective against MRSA because these drugs cannot bind to the bacterial cell wall **(Kreausukon, 2011)**.

According to **Chambers (1997)**, resistance observed to methicillin is attributable to various mechanisms, but the production of a specific PBP-2a, that has a reduced binding affinity for beta-lactamase resistant penicillins and for all other Beta-lactam compounds remains, as before described, the most important mechanism. But, it found that a different chromosomal gene, called *fem*A, which works together with *mec*A gene, is essential for the expression of the methicillin resistance in *S. aureus* (**Unal *et al.,* 1992**; **Chambers 1997**). The *fem*A gene is not found and not expressed in other species of *Staphylococcus* and it appears to be a unique characteristic of *S. aureus* (**Unal *et al.,* 1992**; **Vannuffel *et al.*, 1998**).

Resistance to other antibiotics is also showed to be common phenomenon, principally in hospital-associated MRSA. These organisms are grave nosocomial pathogens, and finding an effective treatment can be challenging (**CFSPH, 2016**). In this regards, MRSA strains have not only resistant to beta-lactam antibiotics family, but can have also capacity to develop special and different resistance mechanisms to a wide range series of antibiotic components, including aminoglycosides family (**Shaw *et al.,* 1993; Schmitz *et al.,* 1999; Choi *et al.*, 2003; Klingenberg *et al.,* 2004; Ardic *et al.*, 2006**) and also to other antibiotics family.

According to authors; in case of amisosides, the major resistance mechanism to this family is generally conferred by an inactivation of antimicrobials by aminoglycoside-modifying enzymes (AMEs) that are decoded by genetic elements (**Vakulenko & Mobashery, 2003; Hauschild *et al.,* 2008; Rahimi, 2016**). The aminoglycoside-6′-Nacetyltransferase/2′′-O-phosphoryltransferase [AAC(6′)/ APH(2′′)] encoded by the aac(6′)/ aph(2′′) gene; aminoglycoside-3′-O-phoshoryltransferase III [APH(3′)-III] encoded by aph (3′)-IIIa gene; and aminoglycoside-4′-O-nucleotidyltransferase I [ANT(4′)-I] encoded by ant (4′)-Ia gene are respectively the most prevalent AMEs among MRSA strains (**Shaw *et al.,* 1993; Schmitz *et al.,* 1999; Fluit *et al.,* 2001; Vakulenko & Mobashery, 2003; Hauschild *et al.,* 2008; Rahimi, 2016**).

Community-associated MRSA strains, which originated outside hospitals, are also frequent in a number of areas. Whilst these organisms have generally been easier to treat, certain have moved into hospitals and have become progressively more resistant to drugs other than beta-lactams. Lately, data obtained from the Centers for Disease Control and Prevention (CDCP) reported that in the US only 59.5% of all healthcare–associated *S. aureus* infections are caused by MRSA (**Palavecino, 2007**). In addition, the MRSA proportion has rapidly augmented from below 5% in the early 1980s to 29% in 1991 (**Kluytmans, 1997**).

1.6.1. Community associated-MRSA (CA-MRSA) and Hospital-acquired-MRSA (HA-MRSA)

MRSA has appeared as a most important causative agent of health care-associated (HA) and community-associated (CA) infections (**Klein, 2007**).

CA-MRSA is considered as an emerging global public health risk, causing mild and life-threatening invasive infections correlated to soft tissues, skin, and respiratory system (**David & Daum, 2010**). A new study by **Wong *et al.,* (2018)** suggested that CA-MRSA accounted for a significant proportion of overall MRSA infections. **Liu *et al.,* (2008)** reported in a prospective study done in the USA that the incidence rate of CAMRSA infections was 243 cases/100,000 population in 2005, while that of health care–associated MRSA infections was 31 cases/100,000 population. CA-MRSA infections were also found among hospitalized patients (**Hidron *et al.,* 2005**), signifying the infiltration of CA-MRSA into hospitals **(Wong *et al.,* 2018**).

The description of the degree and transmission dynamics of MRSA inside the community stays poorly discussed. Numerous studies have confirmed that CA-MRSA strains originating from patients with no antecedent hospital exposure were clonally distinct from hospital endemic MRSA strains (**Vandenesch, 2003; Naimi, 2003**). Indeed, the obvious differences in genotypic and phenotypic between CA-MRSA and hospital-acquired MRSA (HA-MRSA) were reported in anecdotal reports, outbreak studies and case series, which habitually compared few CA-MRSA strains to historical HA-MRSA control isolates from worldwide collections (**Enright, 2002; Mongkolrattanothai, 2003**). For more explanations, the apparent comparison of contemporary cases to historical controls is flawed because one cannot eliminate potential biases due to other factors that possibly will have changed over time (*e.g.* clonal shifts). The importance need for concurrent control is evident (**Alalem, 2008**). According author, a better study design would compare a single series of contemporaneous patients with either CA-MRSA or HA-MRSA disease treated at the same health care location. In addition, the characterization of molecular genetic of MRSA strains combined with better epidemiologic designs will allow the identification of the transmission dynamics of HA-MRSA and CA-MRSA (**Alalem, 2008**).

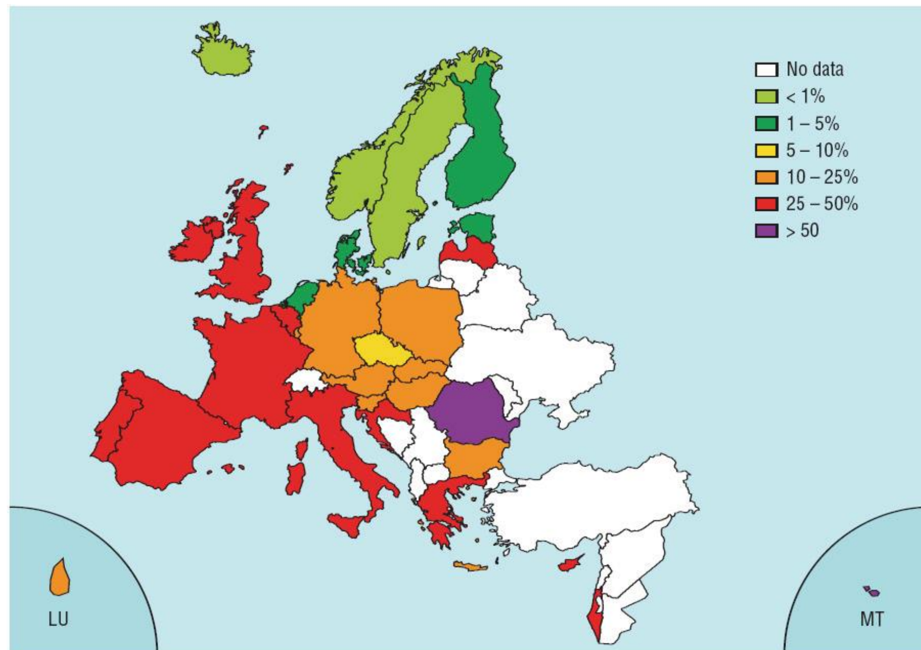

Figure 21: Percentage of MRSA resistance in Europe in 2004: *S. aureus* proportion of invasive isolates MRSA in 2004 (Data from the European antimicrobial resistant surveillance system, EARSS) (**Barcia-Macay, 2007**).

HA-MRSA constitutes 25-50% of clinical isolates all over the world regions (**Diekema *et al.,* 2000**). It has reached more than 40% in Europe (**Lowy, 1998, Lowy, 2003**). In Belgium MRSA accounts for 25-30% (EARSS data, **Figure 21**) (**Barcia-Macay, 2007**). Between 1997 and 1999 MRSA was found to be the most prevalent cause of bloodstream infection, skin and soft-tissue infection and pneumonia in hospital cases in USA, Canada, Latin America, Europe and the Western regions (**Diekema *et al.,* 2001**), see graph below. Today is though that MRSA kills more Americans than HIV, mainly because of hospital practices of no screening for MRSA in blood donors or MRSA asynthomatic carriers entering their presmises (The New York Times, Novembre 14th 2006).

Infections originated of CA-MRSA have been identified habitually in the context of dramatically rising prevalence of MRSA in hospitals with MRSA isolation rates approaching 50% of *S. aureus* infections. Four characteristics, in addition to a lack of nosocomial risk factors, appear to separate differentiate CA-MRSA from endemic hospital MRSA:

- They are not correlated to genotypes endemic in the hospital (**Dufour, 2002; Vandenesch, 2003; Naimi, 2003**).
- They are susceptible to most antibiotics other than Beta-lactams (**Herold, 1998; Adcock, 1998**).
- They carry the type IV *SCCmec* element encoding resistance to the entire class of Beta-lactam antibiotics (**Ma, 2002; Daum, 2002**).

- They carry toxin genes such as Panton-Valentine leukocidin (PVL) and a range of other enterotoxins (**Dufour, 2002; Naimi, 2003**).

In the recent years, **Haran *et al.,* (2012)** showed in study entitled - Prevalence and Characterization of *S. aureus*, Including MRSA, isolated from Bulk Tank Milk from Minnesota Dairy Farms- that of the 2 MRSA isolates, one had a composite genotype profile of MLST ST 5-PFGE USA100-unknown *spa* type, which has been reported among hospital-associated MRSA isolates, while the second isolate carried the MLST ST 8-PFGE USA300-*spa* type t121 genotype, commonly identified among community-associated MRSA isolates. According authors, these results suggest that MRSA genotypes associated with hospitals and community can be isolated from milk at very low rates.
Really, MRSA become a danger bacterium that must be more taken into consideration; because of its great ability to cause severe infections in humans (principally hospitalized) and in animals, which – due mainly to its wide widespread resistance spectrum are difficult to treat.

1.6.2. Methicillin-resistant *S. aureus* (MRSA) in animals

In veterinary medicine, MRSA as well as multi-resistant *S. aureus* strains are occasionally reported (**Sequin *et al.,* 1999; Lee, 2003; Van Duijkeren *et al.,* 2005; Weese et al., 2005; Weese & Rousseau, 2005a; Weese *et al.,* 2006**). Worldwide, an important *S. aureus* strains proportion originated from bovine mastitis (**Moon *et al.,* 2007; Feßler *et al.,* 2010; Hauschild *et al.,* 2012; Haran, 2012; Wendlandt, 2015**), from pigs (**Kadlec & Schwarz, 2009; Kadlec *et al.,* 2010; Schwendener & Perreten, 2011**), and poultry (**Hauschild *et al.,* 2012**) were found to be resistant to methicillin. In cattle dairy, *S. aureus* is a most important causative of chronic or recurring clinical mastitis, and is considered to be a most important contagious mastitis pathogen. The identification of MRSA isolates from bovine mastitis disease has been described by several researchers (**Kaszanyitzky *et al.,* 2007; Lee, 2003; Van Duijkeren *et al.,* 2006; Kwon *et al.,* 2005; Sareyyupoglu *et al.,* 2006; Moon *et al.,* 2007**).

1.7. *Staphylococcus aureus* contagious mastitis pathogen

In the literature, *S. aureus* remains among the most CPS pathogens isolated from cases of bovine mastitis (**Moon *et al.,* 1990; Gianneechini *et al.,* 2002; Olsen *et al.,* 2006; Tenhagen *et al.,* 2006; Piepers *et al.,* 2007; Osman *et al.*, 2009; Ericsson *et al.,* 2009; Malinowski, 2010; Botrel *et al.,* 2010; Smulski *et al.,* 2011; Persson *et al.,* 2011**). As previously described by various studies; in general, *S. aureus* remains one of the most major contagious mastitis pathogens encountered in dairy farms, which is frequently isolated from cows with clinical

(**Bradley** ***et al.,*** **2007; Olde Riekerink** ***et al.,*** **2008; Tenhagen** ***et al.,*** **2008; Persson** ***et al.,*** **2011**) and subclinical mastitis (**Moon** ***et al.,*** **1990; Tenhagen** ***et al.,*** **2006; Bradley** ***et al.,*** **2007; Ferguson** ***et al.,*** **2007**). This pathogen is more concerned because is considered to be one of the most common causatives of bovine mastitis in different areas of the world (**Sutra** ***et al.,*** **1994; Behiry** ***et al.,*** **2011**). **Sutra** ***et al.,*** **(1994)** noted over 20 years ago that this pathogen account responsible for 25 ~ 30% of all intramammary infections (IMI) in the United States in the beginning of 1990s (**Sutra** ***et al.,*** **1994**).

In the United Kingdom, five species of bacteria such as *S. aureus*, *E. coli*, *S. uberis*, *S. dysgalactiae* and *S. agalactiae,* account only for approximately 80% of cases of clinical and sub-clinical in which a pathogen is identified (**Anon, 2001**).

In general, bovine mastitis caused by *S. aureus* stays most frequently subclinical; however, major rates of clinical mastitis incidence are related with this pathogen microorganism. *S. aureus*, generally is considered as contagious mastitis pathogen because it is commonly spread from infected to non-infected cows during milking (**Sears & McCarthy, 2003)**.

Chapter III.

What is Mastitis Disease?

Mastitis disease, generally defined as the inflammation of the mammary gland parenchyma (**Seegers** ***et al.,*** **2003**), characterized by changes in the physical characteristics of the udder or milk (**Nazifi** ***et al.*****, 2011; Hassan** ***et al.,*** **2016**) and most frequently developed in response to an intramammary bacterial infection. They are also considered to be the mainly common and expensive pathology encountered in dairy farming worldwide (**Seegers** ***et al.,*** **2003**).

The classification of mastitis can be referred into three major types: clinical mastitis, sub-clinical mastitis and chronic mastitis (**Anonymous, 2003**). In mastitis type, there is swelling, heat, pain, and indurations observed in the mammary gland and also changes in milk color, clots are present in the milk and there are large numbers of leukocytes in the milk (**Sharma & Maiti, 2010**). However; in sub-clinical mastitis type, there are no gross inflammatory changes in the udder tissue and no changes physical appearance on milk. Only increased somatic cell count (SCC) in the milk, the presence of pathogenic organisms in the milk, and an inflammatory response that can only be detected by screening tests such as California Mastitis Test (CMT),

White Side Test (WST), Surf Field Mastitis Test (SFMT) (**Sachin & Suresh, 2006; Madut *et al.*, 2009**). In case of chronic mastitis type, udder becomes hard due to fibrosis, the quarters may become thickened, firm, nodular and atrophic. The milk may appear as yellowish fluid or white with clots and flakes. Sometimes it may look as green or yellow green and foul smelling (**Chakrabarti, 2007; Hassan *et al.,* 2016**).

1. Subclinical and clinical mastitis

In subclinical mastitis case, only the individual cell count of each cow or the CMT (Califorina Mastitis Test) can identify the presence of this infection (**Schalm *et al.,* 1971**; **Persson *et al.,* 2011**). However, in clinical mastitis case, the infection is characterized by the appearance of visible signs at the neighbourhood, the udder or even the animal. There may be a change in the appearance of the milk (presence of quails, lumps ...), one or more areas swollen, hot, hard or painful and, in cases where more severe, an attack on the general condition of the animal.

2. The host and immune system

Immune system is recognized as a system of host defence including many biological structures and processes within a living organism that protects it against disease. For function correctly, this own system must detect a large variety of agents, known as pathogens, ranging from viruses to parasitic worms, and differentiate them from the organism's own healthy tissue. The immunity can be innate or acquired.

2.1. Protection system of mammary gland

Bovine mastitis is regarded as an important disease touching dairy herds because of the economic losses to dairy producers. This pathology is characterized by a mammary gland inflammatory response caused by physiologic changes and metabolic, trauma or, more frequently, contagious or environmental pathogenic microorganisms (**Oviedo-Boyso *et al.,* 2007**).

The protection of bovine mammary gland parenchyma is naturally assured by innate and specific immune responses (**Sordillo & Streicher, 2002**). But, factors such as physiologic and environmental might compromise the defence mechanism of the mammary gland (**Hopster *et al.,* 1998; Waller, 2000**). According to **Oliver & Sordillo (1988)**, a set of other factors can also involve in the defence mechanism disequilibria such as milking by using machines that can contribute to damage of teat which in turn increases the susceptibility of mastitis-causing pathogen colonization on the one hand and poor housing environment, increased cow densities

per unit and low ventilation can also augment the susceptibility to bovine mastitis on the other hand. But, the lactation stage of a cow is regarded as the major important factor in contributing to increase bovine mastitis susceptibility. It is known that during the periparturient period, the immunity of the udder is compromised due to physiological changes that happen in preparation for lactation adding the authors (**Oliver & Sordillo, 1988**).

In the inflammatory response, the mammary gland immune system is activated to eliminate the pathogen (**Oviedo-Boyso *et al.,* 2007**). According the authors, this mechanism of defence includes anatomical, cellular, and soluble factors that act in coordination and are crucial to the modulation of mammary gland resistance and susceptibility to infection **(Oviedo-Boyso *et al.,* 2007**). During mastitis disease, it is frequent to observe an increased number of somatic cells (neutrophils) in milk **(Oviedo-Boyso *et al.,* 2007**). The migration of neutrophils from the bloodstream to mammary gland tissue happens as a response to pro-inflammatory cytokines such as interleukin 1 beta (IL-1b) and tumor necrosis factor alpha (TNF-a) (**Oviedo-Boyso *et al.,* 2007**). Many cytokines could also increase phagocytic (macrophages and neutrophils) bactericidal activity (**Oviedo-Boyso *et al.,* 2007**). Thereby, cognizance, at the molecular level, of the mammary gland immune response during infection by pathogenic bacteria remains fundamental to the conception of efficacious therapies to eradicate and control bovine mastitis disease **(Oviedo-Boyso *et al.,* 2007**).

2.2. Mammary gland immunology

Little information is available on the host response on intramammary infection. The reason for what we given more importance for information previously reported by **Oviedo-Boyso *et al.,* (2007)** in this topic.

In general, it is well known that the immune system is characterized by its aptitude to recognize and discriminate between foreign invading agents and molecules produced by the organism (**Janeway & Medzhitov, 2002**). The mammary gland accomplishes a set of immunological functions conferring protection; even pre-partum, antibodies secreted in colostrum is produced to protect the new born against infectious agents (**Sordillo *et al.,* 1997**). As aforementioned in several studies, the tissue of mammary gland is protected by two forms of immune defence mechanisms: innate immunity and acquired immunity. The immune systems (innate and acquired) interact closely in an attempt to provide protection against mastitis microorganisms **(Burvenich *et al.,* 2003; Sordillo *et al.,* 1997; Rivas *et al.,* 2002; Sordillo & Streicher, 2002**). Firstly, the innate immune response stimulates the acquired immune response and influences its nature. Secondly, the acquired immune response employs many innate immune effect or

mechanisms to eliminate microorganisms, and its action frequently augments innate antimicrobial activity of immune response.

The effectiveness of these responses defines mammary gland susceptibility or resistance to infection. Innate immunity is predominant in the early phase of infection and normally will mediate by macrophages, neutrophils, natural killer cells (NK) and cytokines. This immunity form recognizes and responds to different pathogens, even if they are invading the mammary gland for the first time. Bacteria have, in particular, different cell wall structures that will recognize by specific plasma membrane receptors. These structures are peptidoglycan (PGN), lipopolysaccharide (LPS), and lipoteichoic acid (LTA), which constitute the pathogen-associated molecular patterns (PAMPs) (**Han *et al.,* 2003; Bannerman *et al.,* 2004**). These PAMPs are recognized by Tolllike receptors (TLRs), which are situated on the cell and endosomal membranes (**Rosenberg & Finlay, 2003**).

According to **Lembo *et al.,* (2003)**, the interaction between the PAMPs and TLRs of immune cells induces production of cytokines and other endogenous mediators that are essential in protection against pathogenic microorganisms. It is now elucidated that TLR4 recognizes the LPS of Gram-negative bacteria (for example *E. coli*) and molecules such as fibrinogen, heat shock proteins, and polypeptides (**Takeuchi *et al.,* 2000; Beutler, 2004**), whereas TLR2 is implicated in recognition of LTA and PGN from Gram-positive bacteria (e.g. *S. aureus*) (**Takeuchi *et al.,* 2000**). On the other hand, TLR9 has been characterized as an endosome-associated receptor able to recognize bacterial DNA and RNA during intracellular infection. According researchers, TLR5 recognizes flagellin, while TLR3 detects double-stranded RNA (**Rosenberg & Finlay, 2003; Wagner, 2004**). Apart from TLRs, recognition of *S. aureus* chemical structures can be mediated through formylated peptide receptors, mannose-binding lectins (MBL), ficolins, and complement molecules. Different authors described that PGN and LTA recognition by MBL and ficolins involves the production of the chemoattractant C5a and deposition of C3b on the surface of *S. aureus*, which facilitates its ingestion and death (**Rosenberg & Finlay, 2003; Akira, 2003; Rooijakkers *et al.,* 2005; Fournier & Philpott, 2005**). Also, the interaction of *S. aureus* protein-A with TNF-a receptor 1 situated in human lung epithelial cells induces an inflammatory response by means of nuclear factor-kB (NF-kB) activation and IL-8 production (**Gomez *et al.,* 2004**).

To gain a better understanding of the mammary gland immune response, the following sections discuss the anatomical, cellular, and soluble factors involved in mastitis (see **Figure 22**) (**Sordillo & Streicher, 2002**).

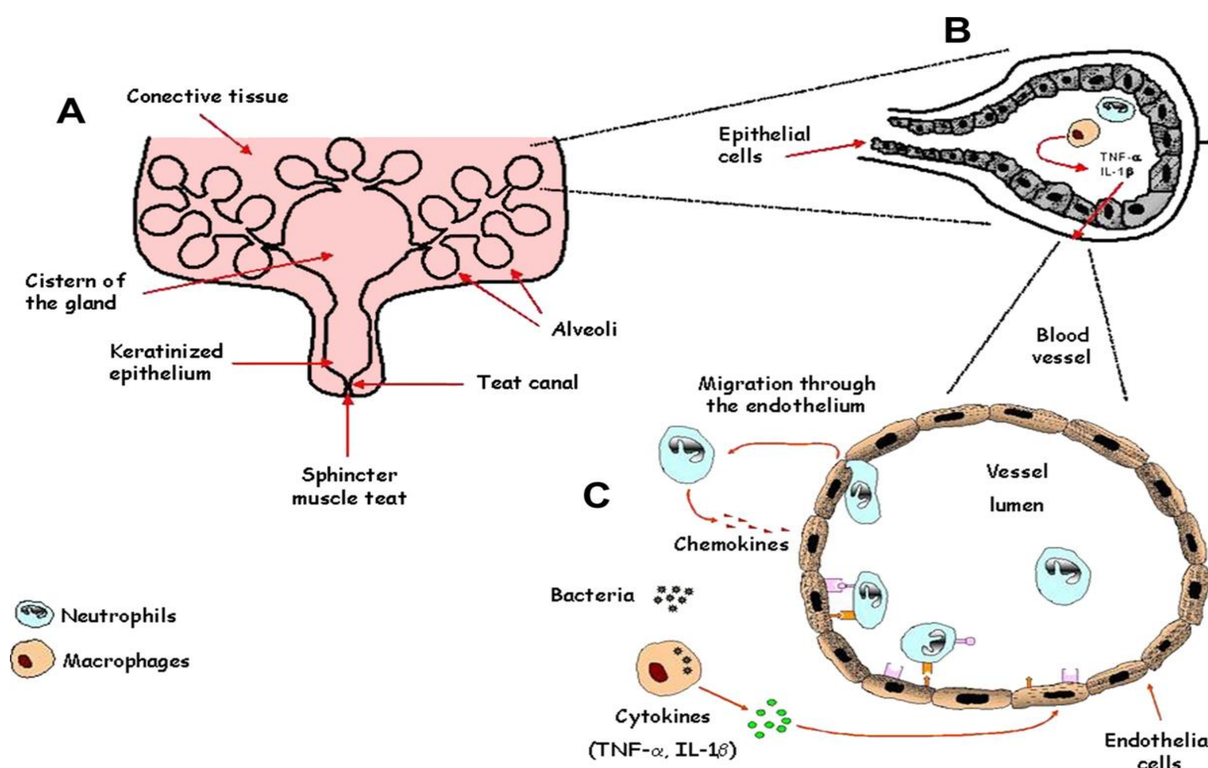

Figure 22: Schematic diagram of the bovine mammary gland showing the most important anatomic factors that act as defense barriers. The teat sphincter muscle represents the first line of defence, whereas the keratinized epithelium of the teat cistern is considered considered the second line. B. Cellular and soluble factors that participate in the innate immune response of the mammary gland. Macrophages located in the alveoli phagocytize bacteria that enter the mammary gland cistern. Activated macrophages release cytokines such as TNF-a and IL-1b. C. Endothelial cells from blood vessels adjacent to alveoli express adhesion molecules in response to pro-inflammatory cytokines; this, in turn, facilitates neutrophil recruitment from the bloodstream to the site of infection in order to eliminate the invading bacteria (**Oviedo-Boyso *et al.,* 2007**).

Several barriers protect mammary gland from infection, including anatomical factors, cellular factors and soluble factors (**Oviedo-Boyso *et al.,* 2007**).

2.2.1. Anatomical factors

The teat canal of mammary gland plays a greatest role in prevention from several microorganisms infections because it forms the first line of defence, for the reason that bacteria should penetrate through this first barrier to cause an intramammary gland (**Figure. 22A**). The essential function of the teat sphincter muscle is to maintain the orifice closed and in that way isolate the interior of the mammary gland). This means that any damage of this structure is associated to an augment in the incidence of mastitis (**Myllys & Honkanen-Buzalski, 1994**). The canal of teat is lined with keratin, which provides an additional physical barrier, preventing bacterial migration in the direction of the mammary gland cistern **(Capuco *et al.*, 1992; Sordillo & Streicher, 2002)**. Esterified and non-esterified fatty acids (myristic, palmitoleic, and linoleic) function as bacteriostatics and are associated with keratin. What is more, certain cationic proteins associated with keratin may bind to pathogenic mastitis microorganisms,

mounting their susceptibility to osmolarity changes (**Miller *et al.,* 1992**; **Paulrud, 2005**). The reason for why, the canal of teat is considered to be an important barrier against intramammary gland (**Oviedo-Boyso *et al.,* 2007**).

2.2.2. Cellular factors

In the literature reviews and also as previously reported by authors (**Oviedo-Boyso *et al.,* 2007**) in this context, the second line of defence consists of neutrophils, macrophages, and lymphocytes that will take place when bacteria penetrate the teat sphincter and the teat canal. These cells regulate both innate and acquired immune responses (**Sordillo *et al.,* 1997; Soltys & Quinn, 1999; Sordillo & Streicher, 2002**).

In mammary gland defence against bacteria causing infection, macrophages of tissue or milk recognize the invading pathogen and initiate the inflammatory response. In this response, pro-inflammatory cytokines induce neutrophil recruitment to the mammary gland (**Figures. 22B,C**) (**Zhang & Issekutz, 2002; Rainard, 2003**).

According aforementioned studies in this regards, milk and healthy tissue of mammary gland contain mainly macrophages, while infected tissue and secretions contain mainly neutrophils. When bacteria are recognized by macrophages cells, these cells release pro-inflammatory cytokines such as TNF-a and IL-1b, stimulating the bactericidal activity of neutrophils and also producing prostaglandins and leukotrienes, which increase the local inflammatory reaction (**Bannerman *et al.,* 2004; Boulanger *et al.,* 2003; Stein *et al.,* 2003**). It is proposed that the epithelial cells of mammary gland play an important role in neutrophil recruitment to the infection site. Adhesion of bacteria to epithelial cells as well as the interaction of bacterial toxins with them induces the synthesis of TNFa, IL-6, and the chemokine IL-8 according to (**Rainard & Riolle, 2003**).

Oviedo-Boyso *et al.,* (2007) described that pro-inflammatory cytokines and chemokines secreted by macrophages or epithelial cells activate in turn the expression of cellular adhesion molecules (E-selectin), intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 by endothelial cells.

This phenomenon causes binding of blood neutrophils to the endothelium, their migration across the epithelial and subepithelial matrix, and their further localization at the infection site or in milk.

For recruitment of neutrophil, participation of chemoattractive molecules such as complement components (C5a and C3a), cytokines (IL-8, IL-12) and even LPS is required (**Zhang & Issekutz, 2002; Nishimura, 2003; Cytokines, 2003; Strindhall *et al.,* 2005**). The increase in

somatic cells count (SCC) (>2 _ 10^5 cell/ml milk) (**Dego *et al.,* 2002**) observed during mastitis disease has its origin in this transendothelial migration and accounts for the importance of endothelial cells in the pathophysiology of this disease.

Neutrophils recruited to the site of infection phagocytize bacteria and produce reactive oxygen species, antibacterial peptides with low molecular weight, and defencing, which eliminate a large variety of pathogens that are known as responsible of mastitis causatives (**Sordillo & Streicher, 2002; Mehrzad *et al.,* 2002; Paape *et al.,* 2003**). Yamaguchi and their collaborators advanced that during the period following parturition, the decrease in neutrophil activity is associated with a higher incidence of mastitis (**Yamaguchi *et al.,* 1999**), if the invading bacteria survive, neutrophil infiltration is replaced after a short period with T and B lymphocytes and monocytes; however, neutrophils remain as the most important type of cell found in chronic mastitis as before suggested by **Rainard & Riolle, (2003)**. Lymphocytes may recognize a set of antigenic structures through membrane receptors that define the specificity, diversity, and the immune system memory characteristics. T lymphocytes are distributed in two principal groups: Tab includes CD4þ (helpers), CD8þ (suppressors) and Tgd. In healthy mammary gland, CD8þ lymphocytes are the prevailing type, while in mastitis CD4þ lymphocytes are predominantly activated by molecular complex recognition. As previously reported by certain studies, this molecular complex is formed between the antigen and major histocompatibility complex class II (MHCII) molecules or by antigen-presenting cells, B lymphocytes, and macrophages (**Park *et al.,* 2004; Ohtsuk *et al.,* 2004**). CD8þ lymphocytes act by eliminating the host cell or by controlling the immune response during an infection caused by bacteria.

In the literature, these lymphocytes have also been regarded as ‘scavengers’ because they eliminate old or damaged cells and their secretions, leading to an augmentation in susceptibility of mammary gland to bacterial infection (**Sordillo & Streicher, 2002; Dosogne *et al.,* 2002; Burchill *et al.,* 2003**). Although Tgd lymphocytes have not been well characterized, they are tightly associated with the epithelial surface, where they destroy damaged epithelial cells as previously reported by **Yamaguchi *et al.,* (1999)**. The main function of B lymphocytes is to produce antibodies against invading pathogens for example bacteria. On the contrary to macrophages and neutrophils, B lymphocytes utilize their membrane receptors to recognize specific pathogens and in the same way as dendritic cells and macrophages, they function as antigen-presenting cells, internalizing, processing and presenting the antigen to CD4þ lymphocytes. Finally, NK cells have capacity to destroy both Gram-positive and Gram-negative bacteria; for this reason, they are fundamental to the prevention of mammary gland infections (**Sordillo & Streicher, 2002**).

2.2.3. Soluble factors

Concerning these soluble factors, **Oviedo-Boyso *et al.,* (2007)** reported that the activity of immune system cells in the bovine mastitis pathophysiology is regulated by pro-inflammatory cytokines that augment bactericidal capacity of macrophage and neutrophil, promote the recruitment of neutrophils towards the infection site (**Figure 22C**), induce the maturation of dendritic cells, and control the acquired immune response (**Alluwaimi & Cullor, 2002; Sordillo *et al.,* 1991; Hornef *et al.,* 2002**).

Recently, a wide variety of cytokines, such as interleukins (IL-1b, IL-2, IL-6, IL-8, IL-12), colony-stimulating factors (CSF), interferon gamma (IFN-g), and TNF-a have been discovered in healthy and infected mammary gland (**Table 4**) (**Sordillo & Streicher, 2002; Alluwaimi, 2004**).

TNF-a is produced by macrophages, neutrophils, and epithelial cells. This cytokine participates in chemotactic activity of neutrophil, because it induces the expression of adhesion molecules by endothelial cells. TNF-a remains the principal cytokine produced during the early stage of infection and is responsible for endotoxic shock in acute mastitis caused by *E. coli* (**Havell, 1989; Slebodzinski *et al.,* 2002; Persson *et al.,* 2003**).

IFN-g is produced by CD4þ/CD8þ lymphocytes and NK cells as a response to mitogenic and antigenic stimuli. It activates the acquired immune response, T lymphocytes and IL-12 production. This cytokine also augments the phagocytic capacity of neutrophils recruited to the mammary gland. Furthermore, it is important in infections caused by viruses (**Strichman & Samuel, 2001; Nonnecke *et al.,* 2003**). IL-1b is produced by monocytes/macrophages and epithelial cells. During the inflammatory response, IL-1b regulates the expression of adhesins by endothelial cells and neutrophil chemotaxis in infections caused by *E. coli*. The role of IL-1b in *S. aureus* infections is important only in the early stages (**Zhang & Issekutz, 2002; Yamanaka *et al.,* 2000**).

IL-2 is produced by CD4þ lymphocytes and was initially described as T cell growth factor. This cytokine regulates the acquired immune response, because it stimulates growth and differentiation of B lymphocytes, increases proliferation of thymocyte, activates NK cells, and induces activation of T lymphocyte. Alterations in IL-2 production cause a capacity decrease in the mammary gland immune response, which contributes to bacterial diseases such as mastitis (**Sordillo & Streicher, 2002; Sordillo *et al.,* 1991**).

IL-6 is a pro-inflammatory cytokine produced by macrophages; it is implicated in acute septic shock during mastitis disease caused especially by *S. aureus* or coliforms. This cytokine facilitates the exchange of neutrophils for monocytes in the mammary gland, which is essential for reduction of the deleterious effects of neutrophils. Also, IL-6 is one of the main regulatory cytokines of acute phase protein synthesis in hepatocytes (**Slebodzinski *et al.,* 2002; Ohtsuka *et al.,* 2001**).

IL-8 is a chemokine produced by monocytes, macrophages and T lymphocytes, as well as epithelial and endothelial cells. IL-8 is actively produced in mastitis caused by *E. coli*, while in mastitis caused by *S. aureus* it is present in low concentrations (**Alluwaimi, 2004; Persson *et al.,* 2003**). IL-12 is a mediator between innate and acquired immunity; it regulates differentiation of T lymphocytes (**Hornef *et al.,* 2002**).

According to **Sordillo *et al.,* (2002)**, the innate immune response is crucial to the control of proliferation of microorganism and to the eradication of pathogens that invade the mammary gland; it is tightly interrelated with the acquired immune response, which is fundamental to establishing a characteristic memory component. Mammary gland also contains non-specific bacteriostatic factors that can act independently or in association with immunoglobulin (Ig). One of these factors is the protein lactoferrin, which is produced by epithelial cells, neutrophils, and macrophages; it binds free iron ions present in milk, making them unavailable to bacteria that need this metal as a growth factor or an iron source (for example *E. coli*, *K. pneumoniae*). However, there are other bacteria that utilize lactoferrin as an iron source (for example *S. agalactiae*) as previously described by **Sordillo & Streicher, (2002)**. The principal function of lactoferrin is to protect the mammary gland against infection caused by coliforms, especially in the involution stage, owing to the activation of phagocytosis and the complement system (**Sordillo & Streicher, 2002; Kai *et al.,* 2002; Lee *et al.,* 2004**).

In various studies, the system of bovine complement is present in serum and milk, and has an important role in the mechanisms of defence of the mammary gland against mastitis. Complement proteins are for the most part produced by hepatocytes, though they are also produced by some monocytes and macrophages in different tissues. Complement molecules are not only needed to recruit neutrophils to the mammary gland, but also to opsonize and kill bacteria. Gram-negative bacteria (for example *E. coli*) are sensitive to complement lytic action, while Gram-positive bacteria (for example *S. aureus*) are resistant; however, all bacteria are susceptible to the opsonizing action of C3b and C3bi. The bactericidal and hemolytic activities of complement are augmented in inflamed mammary gland quarters, and the intensity of these

activities is associated to the inflammatory response. It is well known that hemolytic activity and C3 concentration in milk are higher in mammary gland with mastitis than in healthy mammary gland (**Rainard & Riollet, 2003; Korhonen *et al.*, 2000; Rainard & Poutrel, 2000**).

Table 4: Immune response of bovine mammary gland to different bacteria (**Oviedo-Boyso et al., 2007**).

Bacterium	Type of mastitis	Innate immune response
Staphylococcus aureus	**Clinic or Subclinic/chronic**	**Increase in SCC** **Transit increase in TNF-*a*, IL-1*b*, and C5a concentration** **Increase in IL-12 concentration** **Increase in CD8þ lymphocytes recruitment** **Increase in IgG2 concentration**
Escherichia coli	Clinic	Increase in SCC Increase in TNF-*a*, IL-1*b*, IL-8, IL-12, IFN-*g*, and C5a concentration Increase in LBP, BSA, and sCD14 concentration
Streptococcus uberis	Clinic	Increase in SCC Increase in TNF-*a*, IL-1*b*, IL-8, IL-12, IFN-*g*, pmCD14, and LBP concentration
Serratia marcescens	Clinic	Low concentrations of IL-12, IFN-*g*, pmCD14, and LBP
Klebsiella pneumoniae	Clinic	Increase in SCC Increase in TNF-*a*, IL-1*b*, and IL-12 concentration Increase in IL-8, C5a, LBP, and pmCD14 concentration
Pseudomonas aeruginosa	Clinic	Increase in TNF-*a*, IL-8, IL-12, IL-10, C5a, and LBP concentration

Tumor necrosis factor alpha (TNF-a); interleukin (IL; IL-1b, IL-12, IL-8); interferon gamma (IFN-g); lipopolisaccharide binding protein (LBP); bovine serum albumin (BSA); complement 5a (C5a); plasma membrane receptor (pmCD14); soluble receptor that recognizes the molecular complex LPS-LBP (sCD14); immunoglobulin G2 (IgG2); somatic cell count (SCC).

3. Mastitis in dairy cows

3.1. Aetiology of mastitis

In dairy herds, mastitis can have an infectious or non-infectious aetiology, while the enormous majority of bovine mastitis is of bacterial origin.

Numerous microorganisms, approximately 140 species, have been described as etiological agents associated with bovine mastitis disease in dairy cattle (**Watts, 1988**), being coliforms, streptococci and staphylococci the more often isolated bacteria (**Tenhagen *et al.*, 2006; Piepers *et al.*, 2007; Malinowski, 2010; Smulski *et al.*, 2011**), but other micro-organisms can infect the udder (**Persson *et al.*, 2011**). In fact, the panorama of udder pathogens varies between countries on the one hand and also between types of mastitis, *e.g.* clinical and subclinical on the other hand (**Persson *et al.*, 2011**). For example; in Sweden, a nationwide survey on the microbial aetiology of clinical mastitis was performed in 2002-2003 and revealed that *S. aureus*, *E. coli* and streptococci were the dominating findings according **Ericsson *et al.*, (2009)**. In dairy cows, the *S. aureus* is considered to be one of the most significant organisms associated with clinical and subclinical bovine mastitis (**Moon *et al.*, 1990**). The microbial aetiology of cases

of subclinical mastitis in dairy farms showed that the most frequently isolated bacterial species was *S. aureus* followed by CNS (**Persson *et al.,* 2011**) (**Table 5**).

Table 5: Distribution of bacteriological diagnoses from quarter milk samples from cows newly or chronically infected with subclinical mastitis (**Persson *et al.,* 2011**).

Diagnosis	Newly infected cows (n,%)	Chronically infected cows (n, %)	Total
Staphylococcus aureus	**44 (15%)**	**66 (22%)**	**110 (18.6%)**
Coagulase-negative staphylococci	**51 (18%)**	**46 (15%)**	**97 (16.4%)**
Streptococcus dysgalatae	21 (7.3%)	33 (11%)	54 (9.2%)
Streptococcus uberis	18 (6.4%)	31 (10%)	49 (8.3%)
Streptococcus agalactiae	0	1 (0.3%)	1 (0.2%)
Other streptococci	4 (1.4%)	7 (2.3%)	11 (1.9%)
Enterococci	1 (0.3%)	4 (1.3%)	5 (0.8%)
Arcanobacterium pyogenes	1 (0.3%)	1 (0.3%)	2 (0.3%)
Escherichia coli	7 (2.5%)	10 (3.3%)	17 (2.9%)
Klebsiella spp.	3 (1.1%)	2 (0.7%)	5 (0.8%)
Other coliform bacteria	2 (0.7%)	1 (0.3%)	3 (0.5%)
Other bacteria	2 (0.7%)	1 (0.3%)	3 (0.5%)
Contaminated	60 (21%)	45 (15%)	105 (17.8%)
No growth	72 (25%)	56 (19%)	128 (21.7)
Total	**286**	**304**	**590**

In most aforementioned studies, staphylococci and streptococci are the most common findings in subclinical mastitis (**Gianneechini *et al.,* 2002; Botrel *et al.,* 2010**). Many types of bacteria have been also known as responsible agents of bovine mastitis (**Watts, 1988; Bradley, 2002**). For example, *Klebsiella spp*., *Pseudomonas aeruginosa*., *S. uberis*., *E. coli* and pyogenic bacteria are associated with clinical mastitis form while other microorganisms such as *S. agalactiae*, CNS and *Enterococcus* spp are related with subclinical mastitis form (**Bradley, 2002; Barkema *et al.,* 2009; Awale *et al.,* 2012**).

3.2. Historical perspective of mastitis

Historically, mastitis pathogens have been classified as either ‘‘contagious’’ or ‘‘environmental’’ (**Blowey & Edmondson, 1995**). As previously described, the contagious pathogens are known as organisms adapted to stay alive within the host, in particular inside the mammary gland, and are typically spread from cow to cow at or around the time of milking (**Radostits *et al.*, 1994, Blowey & Edmondson, 1995**). In contrast, the environmental pathogens are best described as opportunistic invaders of the mammary gland, not in particular adapted to survival within the host; typically they enter, multiply, illicit a host immune response and are eliminated (**Geremy, 2003**). Bacteria species such as *S. aureus, S. dysgalactiae* and *S. agalactiae* remains among the major contagious pathogens found whereas *Enterobacteriacae* and *S. uberis* are the environmental pathogens most encountered (**Geremy, 2003**). The line between classic contagious and environmental behaviour of mastitis pathogens has become blurred (**Geremy, 2003**). Persistent infection with both *E. coli* (**Hill *et al.*, 1979; Lam *et al.*, 1996; Dopfer *et al.*, 1999; Bradley & Green, 2001**) and *S. uberis* has been reported (**Todhunter *et al.*, 1995; Zadoks *et al.*, 2003**). Certain studies reported in the Netherlands stated that 9.1% (**Lam *et al.*, 1996**) and 4.8% (**Dopfer *et al.*, 1999**) of clinical *E. coli* mastitis recurred in a quarter, as well in the UK, **Bradley & Green (2001).**

Other study reported of clinical mastitis identified *E. coli* as being the most widespread cause of recurrent clinical mastitis, with 20.5% of all cases being recurrent, as confirmed by DNA fingerprinting (**Bradley & Green, 2001**). If these ‘environmental’ pathogens can exist in the mammary environment for prolonged periods, it is likely that contagious spread will occur between cows and this has been suggested for *Strep. Uberis* infections (**Zadoks *et al.*, 2003**).

4. Bovine mastitis and its impact on the dairy industry

Mastitis pathology represents one of the most widespread diseases encountered in dairy cows. In the world, mastitis is an endemic disease and happens to be the most prevalent and most expensive disease touching dairy herds (**Miller *et al.,* 1993; Halasa *et al*., 2007**).

In the dairy industry worldwide, this pathology remains also the most costly diseases with estimated losses of about 2 billion dollars per year, for example, in the United States alone (**Gruet *et al.,* 2001**). These considerable economic losses caused by clinical or subclinical mastitis in dairy farms are due to the several factors such as rejected milk, reduced milk production and quality, drug costs, veterinary expenses, materials and investments, early culling, and increased laboratory costs (**Gruet *et al.,* 2001; Halasa *et al.,* 2007**). Although, the relative costs of these factors described might differ between countries and also between

regions, but it appears that the economic principles behind them are the same (**Halasa *et al.,* 2007**).

Mastitis is a pathology that affects quality of milk directly in the technical characteristics and the hygienic quality of the milk, and indirectly through the intrinsic milk quality (**Hogeveen & Lankveld, 2002**); especially through physical and chemical changes of the milk composition accompanied often with pathological changes generally observed in the udder tissues (**Radostits *et al.,* 2000**) and in return, affects the economy of dairy industry. This disease can cause a number of variations in essential elements of milk such as a decline in potassium level and an increase in lactoferrin. It also results in decreased casein rate, the major protein found in milk in cattle dairy. As most calcium encountered in milk is related with casein; therefore, the disruption of casein synthesis contributes to lowered calcium in milk. The milk protein continues to undergo further deterioration during processing and storage (**Jones & Bailey, 2010**). Milk from cows suffering with mastitis also has a higher somatic cell count (**Kandasamy *et al.,* 2011**).

5. Consequences of mastitis: An economic framework

The estimation of economic impact of mastitis has been proposed by several methods, including estimates for the entire industry and for individual herds (**Gill *et al.,* 1990; Schepers *et al.,* 1991; DeGraves & Fetrow, 1991; Van Eenennaam *et al.,* 1995; Allore & Erb, 1998**). All estimates suggest that mastitis is a costly production disease touching dairy herds, but estimates differ greatly because they are formulated using different estimation methodologies, different sources of loss, and different origins of data (**Schepers & Dijkhuizen, 1991**).

For more elucidation, the economic consequences that might be associated to clinical or subclinical mastitis are particularly due in the most cases to a variety of factors such as losses in milk production, discarding abnormal milk and milk with held from cows treated with antibiotics, treatment, culling, changes in product quality (in instance degrading of milk quality and price due to high bacterial or SCC, problems related to antibiotics residues in milk and its products), risk of other diseases and lost future income that results from culling (**Schepers & Dijkhuizen, 1991; Harmon, 1994; Allore & Erb, 1998; Halasa *et al.,* 2007; Abdel-Rady & Sayed, 2009**). As previously described by various studies below, the sources of losses and associated costs generated by this disease can be divided among the following factors:

- Substantial milk production losses,

- Discarded milk (discarding abnormal milk),

- Product quality,

- Mastitis veterinary services fees,

- Diagnostics,

- Use of Drugs,

- Increased labour,

- Materials and investments,

- Risk of other diseases,

- Economics of Resistance phenomenon and

- Culling and lost future income that results from culling (costs of replacement heifers).

According to **Halasa *et al.,* (2007)**, the relative costs of these all factors could be different among countries and also among regions; but, the economic principles behind them are the same and will be explained below.

5.1. Substantial milk production losses

In clinical and subclinical mastitis form there is a considerable loss in production of milk. In aforesaid studies on clinical mastitis, production losses due to this mastitis form have been esteemed (**Houben *et al.,* 1993; Hortet & Seegers; 1998; Grohn *et al.,* 2004**). Although, production losses of milk observed to be associated to subclinical mastitis are generally considered to be a direct log-linear relationship between SCC and test-day records (**Bartlett *et al.,* 1990; Miller *et al.,* 1993**). However, the milk production does not improve after complete recovery of subclinical mastitis as previously found by **Rose *et al.,* (2003)**. In consequence the assumed log-linear relationship could underestimate production losses due to subclinical mastitis **(Halasa *et al.*, 2007**).

5.2. Discarded milk (discarding abnormal milk)

According to **Halasa *et al.,* (2007)**, economic damage associated to rejected milk stays comparable with that from decreased milk production. However, there is one difference: the discarded milk is actually produced by the cows, which means that feeding costs for that quantity of milk must be taken into consideration in the calculations. The economic damage of 100 kg of discarded milk is consequently larger than for 100 kg of decreased production. Although it is not advisable from a veterinary point of view, discarded milk is often fed to

calves instead of milk replacer, in this manner thereby saving the cost of that milk replacer **(Halasa *et al.,* 2007)**.

5.3. Product quality

This factor takes into consideration two quality parameters of meat and milk. Mastitis is a disease that does influence the quality of milk, but this disease has no effects on the quality of meat (**Hoblet & Miller, 1991; Berry *et al.,* 2004; Halasa *et al.*, 2007**). In milk case, mastitis cause changes in milk quality, thus leads to less efficient processing of milk and could result in products with less valuable properties (**Ma *et al.,* 2000; Santos *et al.,* 2003; Halasa *et al.,* 2007**). The associated economic damage is difficult to calculate whereas the estimation of the direct effect of this economic damage for the individual dairy farmer is even more difficult to assess. The only modifications in quality of milk that have a direct impact, and can be esteemed, are the factors that are part of the milk payment system, for example, bacterial count and somatic cell count. Bacterial count and/ or somatic cell count do vary with the mastitis status of a cow and therefore, in almost countries in the world, there is a regulatory limit (payment schemes or bonus systems) for bulk milk bacterial count and bulk tank somatic cell count (BTSCC). In instance, BTSCC can augment strongly due to a subclinical mastitis case (**Pyorala *et al.,* 1987**), which will have economic consequences (**McInerney *et al.,* 1992**). Besides BTSCC and bacterial count, almost milk payment schemes test for antibiotic residues. Although the mastitis in itself does not affect growth inhibition, the antibiotics utilization in treatment of mastitis disease does increase the risk of penalties. According to **Beek *et al.,* 1992,** numerous countries and milk processors followed different rules utilizations for antibiotic residues suspected in the milk, but the economic consequences associated to antibiotic residues found in the milk can be substantial.

5.4. Mastitis veterinary services fees

In many countries, besides delivering drugs; the veterinarian might have to spend time on diagnosis of a (clinical) mastitis case (**McNab & Meek, 1991**). Veterinary services can be obligatory for each (clinical) mastitis case, if required by (national) nationwide legislation, or is only provided upon request by the farmer **(Halasa *et al.,* 2007)**.

5.5. Diagnostics

According certain authors, all costs of diagnostics that appears to be appropriated to mastitis must be included in the calculations, for example costs of technicians and bacterial cultures (**Al lore & Erb, 1998; Zepeda *et al.,* 2000; Halasa *et al.,* 2007**).

5.6. Use of drugs

The necessary use of drugs to treat infected animals is appeared as a direct cause of economic damage because of their costs. The variation of costs of drugs remains different between countries, depending on the infrastructure and the legislation of each country (**Halasa *et al.,* 2007**). Concerning the management of antimicrobials use in dairy herds in different countries is further described below in the management of mastitis in the study farms axis.

5.7. Increased labour

The interpretations of labour costs due to mastitis are difficult to explain. Opportunity costs of labour can be different from farm to other. According to **Halasa *et al.,* (2007)**, if the labour is external, then the cost of labour for the time that has been utilized to prevent mastitis is quite easy to calculate (hours x hourly wage). If the labour comes from the farmer's free time, the opportunity costs are considered zero. However, if because of mastitis the farmer spends less time on other management tasks, the opportunity costs are the decrease in income due to skipping these tasks (**Halasa *et al.,* 2007**).

5.8. Materials and investments

Management of mastitis includes the utilization of a variety of materials and commodities that cost money. According to **Halasa *et al.,* (2007)**, this management needs of a range of materials that can either be renewable (such as disinfectants and drugs could be seen as specific types of renewable materials) or non-renewable (such as a new milking parlour). In dairy farms, the buying of renewable materials has short term economic consequences and the associated cost of these materials can easily be calculated; while the buying of non-renewable materials has long-term consequences. Costs linked to purchase have to be divided over different years by depreciation. Moreover, because capital is tied up by such purchases interest rates must be calculated as well. In the end most of materials that are non-renewable demand maintenance and this also generates costs (**Halasa *et al.,* 2007**).

5.9. Other diseases

As described above, all factors advanced such as veterinary services, diagnostics, use of drugs, substantial milk production losses, discarded milk, labour, product quality, materials and investments, and culling are represented the economic consequences of clinical and subclinical mastitis (**Halasa *et al.,* 2007**).

Besides these factors direct costs, cows suffering from mastitis are a constant source of infection due to the shedding of bacteria (**Zadoks *et al.,* 2002; Swinkels *et al.,* 2005**). There might also be an association between mastitis and other cattle diseases such as previously lead by others various studies (**Peeler *et al.,* 1994; Kossaibati & Esslemont, 1997; Grohn *et al.,* 2003; Halasa *et al.*, 2007**). The causal relation, however, is difficult to determine. When the risk of other diseases is augmented by mastitis, the economic damage of other disease cases attributable to mastitis can be seen as economic damage due to mastitis. According to **Halasa *et al.,* 2007,** this damage stays very hard to determine for the reason that because the interactions between a range of diseases are hard to establish. Maybe this would be a good topic for more research according authors.

6. Control Strategies of mastitis

In the dairy industry, mastitis in both subclinical and clinical forms remains an expensive, frustrating and extremely complex pathology that results in a marked (noticeable) reduction in the quality and quantity of milk (**Harmon, 1994; Abdel-Rady & Sayed, 2009**). Over the world, subclinical mastitis is regarded as a major problem affecting dairy animals. According to **Ramachandrainh *et al.,* (1990)**, subclinical mastitis causes enormous losses for breeders and consequently influences the national income of the country.

Preventive control strategies of mastitis could take account of a variety of parameters for preventing mastitis infections such as:

Milking hygiene,

Milking equipment maintenance,

Types of housing and bedding, and

Nutrition **(Allore & Erb, 1998)**.

According to certain researchers, an effective mastitis control program includes rapid identification and treatment of clinical mastitis cases, entire herd antibiotic dry cow therapy, post milking teat disinfection, culling of cows with chronically infected mastitis, and routine maintenance of milking machines (**Natzke, 1981; Dodd, 1983; Smith, 1983; Oliver & Mitchell, 1984; Harmon, 1996**).

The amelioration of the National Mastitis Council recommendations (NMC) for milking hygiene in some countries might lead to positive consequences for more mastitis prevention. In this context, **Allore & Erb (1998)** modelled, for example, the NMC for milking hygiene, which included forestripping, predipping all teats, cleaning and drying with a single-use paper towel, and postdipping. Prevention decreased the risk of new IMI by 54, 7, and 87%, respectively, for IMI caused by *S. agalactiae*, *Streptococcus* spp. other than *S. agalactiae*, and *S. aureus* [modified from **Drechsler *et al.,* 1993**. Prevention decreased the risk of new IMI by 38% for IMI caused by CNS and by 0% for IMI caused by *E. coli* [modified from **Oliver *et al.,* 1984**. The pathogen-specific form of prevention for mastitis caused by *E. coli* is vaccination. This control strategy was set to decrease the risk of new infection caused by *E. coli* by 80% (**Gonzales *et al.,* 1989**).

Bacteriologic cure rates for lactation therapy were set to 75, 82, 56, 62, and 22% for IMI caused by *S. agalactiae*, *Streptococcus* spp., other than *S. agalactiae*, *S. aureus*, CNS, and *E. coli*, respectively according to **Wilson (1996**).

Recently, the obtained results by **Abdel-Rady & Sayed, (2009)** threw the light on the epidemiology of subclinical mastitis in Assiut villages and provided an importance of the CMT for diagnosis of subclinical mastitis due to it is a reliable, easy, rapid and cheap tool helping in diagnosis and controlling the disease because it directs attention to individual mammary quarter that is secreting milk of high somatic cell count (SCC). In this regards, **Abdel-Rady & Sayed, (2009)** confirmed that programs for control of subclinical mastitis can be planned around the routine examination of all lactating cows, and therefore early treatment can be applied towards positive cases rapidly for preventing their conversion towards clinical form among dairy cows and for protecting the herd health, milk hygiene and consequently the consumer health. Finally, several aforementioned authors confirmed also that contagious mastitis caused by this pathogen may effectively be controlled by implementation of a mastitis control program including rapid identification and treatment of clinical mastitis cases, post milking teat disinfection, whole herd antibiotic dry cow therapy, routine maintenance of milking machines and culling of chronically infected cows (**Natzke, 1981; Dodd, 1983; Smith, 1983; Oliver & Mitchell, 1984; Harmon, 1996**).

Lately, one of among modern strategies followed for controlling mastitis includes a combination of post-milking dipping and dry cow therapy associated accompanied with good veterinary practice of administrating antimicrobial compounds in order to prevent or treat new infections on farms (**El Behiry *et al.,* 2012**). But, it appears that this strategy is not always been fully successful according researchers. The failure of these programs to control bovine mastitis

can be partly attributed to teat disinfectants and/or antibiotics which do not afford sufficient protection against multiple pathogens that cause mastitis, particularly *S. aureus* and CNS as described by some previous studies (**Gruet *et al.,* 2001; Pyörälä & Taponen, 2009**). Dairy producers should identify the causative mastitis pathogens in their herd in order to develop an effective control program (**Kreausukon, 2011**). Inadequate mastitis control programs can lead to lower milk yields; poor udder health, milk quality, and milk composition; low cheese yield; shorter shelf-life; and a decrease in profitability to both producers and processors and consequently influences the national income of the country.

When these strategies are not successful for preventing and controlling mastitis within dairy herds, farmers and producers refuge directly to use antimicrobials for treat cows with mastitis and for eradicate definitively maicroorganisms caused mastitis infections. But the problems that involved more often is how use these antimicrobials by farmers and producers?

7. The management of mastitis in the studied farms

Dairy producers should identify the causative mastitis pathogens in their herd in order to develop an effective control program (**Kreausukon, 2011**). The identification of the pathogens that cause mastitis by using milk microbiological culture remains a useful tool in this sense (**Ferguson *et al.,* 2007**).

Certain farms reported that they treated cows with clinical signs of mastitis with antimicrobial agents. The majority of the farmers treated the cows doubted to have mastitis when they found abnormal milk (**Kreausukon, 2011**). Approximately 40% treated cows with high SCC. **Thomson *et al.,* (2008)** reported that 37% of veterinarians, in Finland, based the diagnosis of acute mastitis on clinical signs, and veterinarians used bacteriological examination to target the treatment in the majority of cases (73%) of mastitis with subclinical form while in the USA, on numerous farms, detection, diagnosis and administration of treatment for mild and moderate cases of clinical mastitis are the responsibility of farm personnel and veterinarians are often consulted only when a case becomes life-threatening **(Kreausukon, 2011)**. In case of Pennsylvania, 50% of dairy farms surveyed, in 113 dairy herds, maintained antibiotic treatment records, and only 21% had a written plan for treating sick animals (**Sawant *et al.,* 2005**). Poor knowledge and insufficient record keeping about drug withdrawal periods among producers were important factors leading to drug residues in milk (**Kaneene & Ahl, 1987**).

Evidence based veterinary medicine (EBVM) is an application of the principles of evidence based medicine, utilized by physicians, to clinical decision making for animals receiving veterinary care (**Ruegg, 2010**). According to author, the application of concepts of EBVM to

mastitis therapy has the potential to improve treatment protocols and results of better therapeutic outcomes (**Ruegg, 2010**).

Kreausukon, (2011) showed in a study that with respect to the duration of therapy 69.1% of the farms treated the cows for 3 to 4 days, 16.4% treated the cows for 1 to 2 days, and 14.5% treated for more than 4 days. About one-third of the farms included in this study signalized they treated the cow up to the moment that the milk has a normal appearance again. As previously described in a study done on dairy herds in Brandenburg, Germany, approximately 45% of farmers treated cows 3 to 4 times for each case of mastitis and 22% of farmers administered more treatments or augmented the period of treatments (**Tenhagen *et al.,* 2006**). Although supported by a range of studies that showed increasing success of treatment when treatment is extended (**Oliver *et al.,* 2004**), this practice, conflicts with the legal obligation to preferentially utilize a drug as laid down in the description given by the pharmaceutical company, which was the basis of the licensing decision **(Kreausukon, 2011)**. Exceptions from this "rule" have to be only made when justified by the outcome of theclinical examination. In Finland case, **Thomson *et al.,* (2008**) reported that the prescribed duration of treatment ranged from 1 to 8 days, and the median duration was 4 days. In general, **Ruegg** reported that the duration of antibiotic treatment is kept as short as possible to minimize the economic losses related with milk discarded. Discarded milk remains one of the majority proportions of expense related with treatment of clinical mastitis (**Ruegg, 2010**).

The separation of cows with mastitis from the milking cows appears different between studied farms. In this context, **Tenhagen *et al.,* (2006)** reported that in Brandenburg (in Germany), 20% of farms did not separate sick and treated cows from the milking cows, and two farms reserved those cows among the herd mates without marking them. Moreover, **Kreausukon, (2011)** reported that half of the study farms left the cows that had been treated for mastitis in the milking herds, only 45% separated those animals, and 5% used both options.

In aforementioned studies, researchers showed that the utilization of antimicrobial agents on farms always poses the risk of the milk becoming contaminated with antibiotic residues (**Zwald *et al.,* 2004; Sawant *et al.,* 2005**). According to **Kreausukon, (2011)** quality assurance programs on dairy farms request the identification and /or separation of cows receiving antibacterial compounds for guarantee that milk that is delivered to the dairy factory will not be contaminated with antimicrobial residues. In this away, physically separating treated cows from healthy milking cows, marking them visibly, and milking them last in separate milking units are effective in preventing drug residues in milk (**Sawant *et al.,* 2005**).

8. Use of antibiotics in dairy cows

Antibiotics are drugs that have antibacterial properties. A number of antibacterial classes can share similar characteristics to others and are active against similar pathogens, while other antibacterial classes have very specific properties and may be active against different organisms. Based on their action mechanism they are classified into a number of different classes as follows (**Table 6**) (**HPRA, 2019**):

Table 6: Main antibiotic classes used in dairy cows.

Main antibiotic classes	Mode of action	Main uses (OIE, 2015; HPRA, 2019)
Penicillins	Act on bacterial cell walls	Treatment of septicaemias, respiratory and urinary tract infections.
Cephalosporins	Act on bacterial cell walls	Treatment of septicemias, respiratory infections, and mastitis.
Tetracyclines	Inhibit protein synthesis in bacteria	Treatment of many bacterial and other diseases.
Potentiated sulphonamides	Inhibit synthesis of folate in bacteria	Treatment of a wide range of diseases (bacterial, coccidial and protozoal infections).
Macrolides	Inhibit protein synthesis in bacteria	Treatment of respiratory and other infections.
Amphenicols	Inhibit protein synthesis in bacteria	Treatment of respiratory infections.
Aminoglycosides	Inhibit protein synthesis in bacteria	Treatment of septicemias, respiratory infections, and mastitis.
Fluoroquinolones	Inhibit DNA synthesis in bacteria	Treatment of septicaemias, respiratory and enteric diseases.

Antibiotics behave in precise ways when they enter the animal's bloodstream; some are distributed evenly throughout the body, while others are concentrated in specific locations for example the lungs. Antibiotics are subject to prescription control; the veterinary practitioner is best placed to prescribe the most appropriate antibiotic for the disease in question.

9. Precautions in use of antibiotics in dairy cows

According to the Health Products Regulatory Authority (HPRA), the dose of the antibiotic stated on the product labelling has been approved by (HPRA) the regulatory authority concerned. This is usually done on the basis of pre-clinical studies as well as clinical field studies and therefore follows a detailed scientific and evidence-based evaluation of the quality, safety and efficacy of each individual medicine. Deviations from those conditions of use

established by may invalidate the expected performance of the veterinary medicine under field conditions. In particular, users are reminded (**HPRA, 2019**):

1. Utmost care is needed to ensure that cows that have been treated with an antibiotic are clearly marked and identified, to prevent milk being inadvertently used for human consumption before residues have depleted sufficiently to levels below the permitted limit.
2. To strictly adhere to the withdrawal periods stated on the product labelling and package leaflet.
3. That the use of an animal remedy at a dose above that stated on the product labelling might be expected to prolong the time needed by the animal to clear residues of the medicine beyond that given as the withdrawal period for the product.
4. That the use of an antibiotic concurrently with another medicine, e.g. a steroid or a different antibiotic, might lead to an interaction between both medicines, such that the withdrawal periods of both products is not sufficient to ensure that residues will be the legal limits at the end of the withdrawal periods.
5. That some antibiotics *e.g.* gentamicin have a prolonged withdrawal period (214 days for meat). Re-treatment of those animals during this period may result in an accumulation of drug residues necessitating an even longer withdrawal period.
6. Antibiotics carry particular risks for antimicrobial resistance when used off-label. Such use is restricted, by law, to veterinarians and only under specific circumstances.
7. The antibiotic screening tests routinely used by creameries might not be calibrated precisely in line with the European residue limits set for public health and may be exquisitely sensitive for certain classes of antibacterial drugs (for example penicillins and cephalosporins) while being less sensitive to other drugs. This can result in the withdrawal period stated on the product label to be insufficient to guarantee that the milk will pass the creamery standards for trading purposes (even though acceptable for public health on the basis of consumer health standards).
8. That milk from treated cows which contains antibiotic residues is expected to transfer antimicrobial resistance if fed to calves. Ideally, such milk should be disposed of into the slurry tank, where it is denatured and diluted.

Material and Methods

1. Bacterial strains studied

A series of Fifty-two *Staphylococcus* isolates isolated from milk samples received from clinical and subclinical mastitis bovine at the laboratory of Microbiology and Infectious Diseases of the Veterinary Clinics Department at Institute of Sciences Biomedical Abel Salazar (ICBAS), University of Porto (Portugal) between the years of 2003, 2004, 2007, 2008 and 2017 were used and included in this thesis work in order to maintain and force the scientific connection between farms and research laboratory. The availability of strains has originated from 37 farms situated in Northwest of Portugal [farm 1 (3 isolates), farm 2 (1 isolate), farm 3 (1 isolate), farm 4 (1 isolate), farm 5 (1 isolate), farm 6 (1 isolate), farm 7 (1 isolate), farm 8 (2 isolates), farm 9 (1 isolate), farm 10 (1 isolate), farm 11 (1 isolates), farm 12 (1 isolate), farm 13 (1 isolate), farm 14 (1 isolate), farm 15 (1 isolate), farm 16 (1 isolate), farm 17 (1 isolate), farm 18 (1 isolate), farm 19 (1 isolate), farm 20 (2 isolates), farm 21 (1 isolate), farm 22 (1 isolate), farm 23 (3 isolates), farm 24 (2 isolates), farm 25 (2 isolates), farm 26 (1 isolate), farm 27 (35 isolate), farm 28 (2 isolates), farm 29 (3 isolates), farm 30 (1 isolate), farm 31 (4 isolates), farm 32 (1 isolate), farm 33 (1 isolate), farm 34 (1 isolate), farm 35 (1 isolate), farm 36 (1 isolate), farm 37 (2 isolates)]. All these isolates belong to the microorganisms' collection of the laboratory of Microbiology and Infectious Diseases, Department of Veterinary Clinics of the Institute of Biomedical Sciences Abel Salazar of the University of Porto, Porto, Portugal, and SVAExpleite, Ldª, Fradelos, Portugal. Information about these isolates can be found in **Table 7**.

Table 7: Herds and year of collection of the *S. aureus* isolates used in the study.

Herd	Isolates	Year
1	1 / 2 and 3	2003 / 2008
2	4	2003
3	5	2004
4	6	2003
5	7	2003
6	8	2003
7	9	2004
8	10 and 11	2003
9	12	2003
10	13	2004
11	14	2003
12	15	2003
13	16	2003
14	17	2004
15	18	2003
16	19	2004
17	20	2007
18	21	2008
19	22	2008
20	23 and 24	2008
21	25	2008
22	26	2008
23	27, 28 and 29	2017
24	30 and 31	2017
25	32 and 33	2017
26	34	2017
27	35	2017
28	36 and 37	2017
29	38, 39 and 40	2017
30	41	2017
31	42, 43, 44 and 45	2017
32	46	2017
33	47	2017
34	48	2017
35	49	2017
36	50	2017
37	51 and 52	2017

2. Isolation of Staphylococcus isolates

The isolation of *Staphylococcus* isolates from milk samples was performed by using conventional and classical bacterial methods: culture of milk samples on blood agar and on Mac Conkey media.

3. Diagnostic tests for identification of *S. aureus*

Bacterial strains were firstly identified and recognized as *S. aureus* by following conventional microbiological methods, including, use presence of haemolysis after overnight incubation at 37°C on Columbia ANC + 5% sheep blood (BioMérieux, Marcy l'étoile - France). Haemolysis was recorded as haemolysis, double haemolysis, and negative (no haemolysis). Moreover, colonial and microscopic morphology examination and tube tests were performed to demonstrate coagulase and catalase activities. The coagulase test was performed with rabbit plasma following the recommendations of the manufacturer (BioMérieux). Results were recorded after 24 h of incubation at 37°C. Weak coagulase activities were recorded as positive. The isolates were further tested with the commercial Slidex Staph Plus. The confirmed *S. aureus* isolates were stored at -20°C.

3.1. Columbia Agar + 5% Sheep Blood + CNA

Selective culture medium with sheep blood, colistin, and nalidixic acid for isolation of Gram-positive cocci.

Figure 23: Selective culture medium with sheep blood, colistin, and nalidixic acid.

Columbia Agar with 5% sheep blood and CNA (colistin + nalidixic acid) is a solid selective culture medium for the isolation of pathogenic gram-positive cocci from clinical and non-clinical specimens. For more blood agar medium detailed information consult annex part.

3.2. Gram Staining Procedure/Protocol

Gram staining method, the most important procedure in Microbiology, was developed by Danish physician Hans Christian Gram in 1884. Gram staining is still the cornerstone of bacterial identification and taxonomic division.

This differential staining procedure separates most bacteria into two groups on the basis of cell wall composition (**Figure 24**):

1. Gram-positive bacteria (*thick layer of peptidoglycan-90% of cell wall*)- **stains purple**.
2. Gram-negative bacteria (*thin layer of peptidoglycan-10% of cell wall and high lipid content*) –**stains red/pink**.

3.2.1. Smear preparation

Fix material on a slide with methanol or heat. If the slide is heat fixed, allow it to cool to the touch before applying the stain.

1- Flood air-dried, heat-fixed smear of cells for 1 minute with **crystal violet** staining reagent. Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

2- Wash slide in a gentle and indirect stream of tap water for 2 seconds.

3- Flood slide with the mordant: **Gram's iodine.** Wait 1 minute.

4- Wash slide in a gentle and indirect stream of tap water for 2 seconds.

5- Flood slide with **decolorizing agent (Acetone-alcohol decolorizer)**. Wait 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.

6- Flood slide with a counterstain, **safranin**. Wait 30 seconds to 1 minute.

7- Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.

8- Observe the results of the staining procedure under oil immersion (100x) using a Bright field microscope.

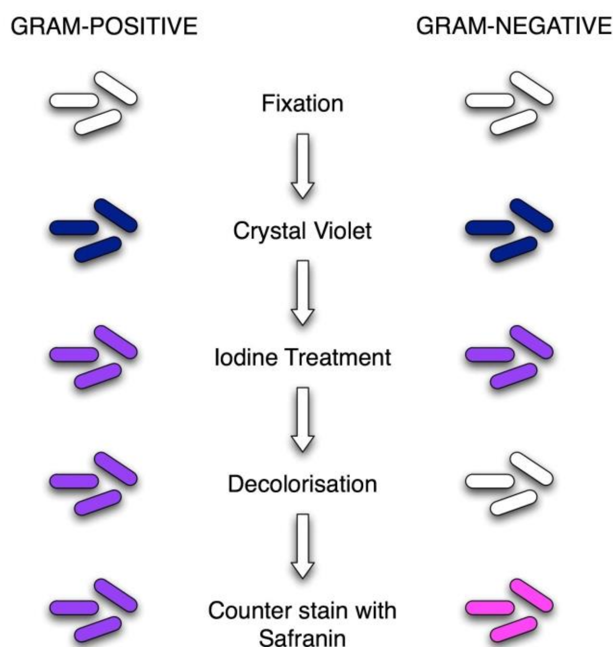

Figure 24: Differentiation principle of Gram Staining.

3.3. Catalase test

The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2$ + Catalase → $2H_2O + O_2$). This reaction is evident by the rapid formation of bubbles. There are many applications and method variations of the catalase test. These include the slide or drop catalase test, the tube method (**Reiner, 2010**).

Place a microscope slide inside a petri dish. Using a sterile inoculating loop or wooden applicator stick, collect a small amount of organism from a well-isolated 18- to 24-hour colony and place it onto the microscope slide. Do not mix, immediately cover the petri dish with a lid to limit aerosols and observe for immediate bubble formation (O_2 + water = bubbles).

The use of a petri dish is optional as the slide catalase can be properly performed without it. However, to limit catalase aerosols, which have been shown to carry viable bacterial cells, the use of a petri dish is strongly recommended (**Reiner, 2010**).

Positive reactions are evident by immediate effervescence (bubble formation) (**Figure 25**). The use of microscope to observe weak positive reactions are sometimes recommended. If using a microscope, place a cover slip over the slide and view under 40x magnification. No bubble formation (no catalase enzyme to hydrolyse the hydrogen peroxide) represents a catalase-negative reaction (**Figure 25**).

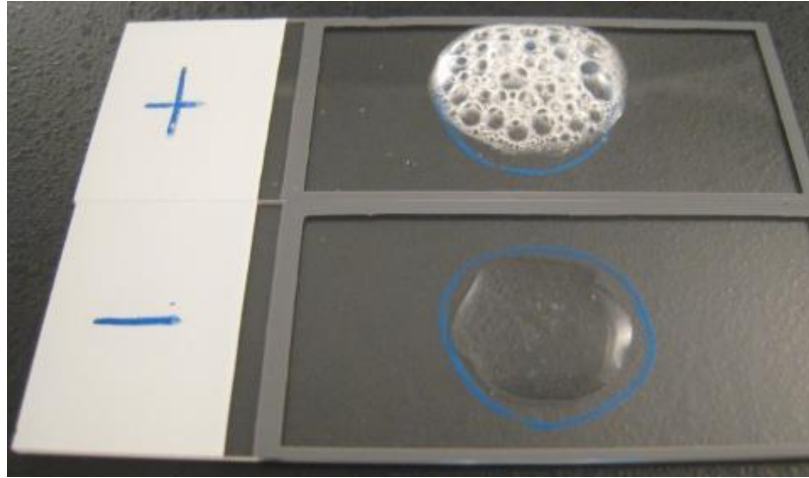

Figure 25: Slide catalase test results. (Top) The positive reaction was produced by *S. aureus*; (bottom) the negative reaction was produced by *Streptococcus pyogenes*.

Figure 26 showed evident positive reactions by immediate effervescence (bubble formation) obtained by using tube method.

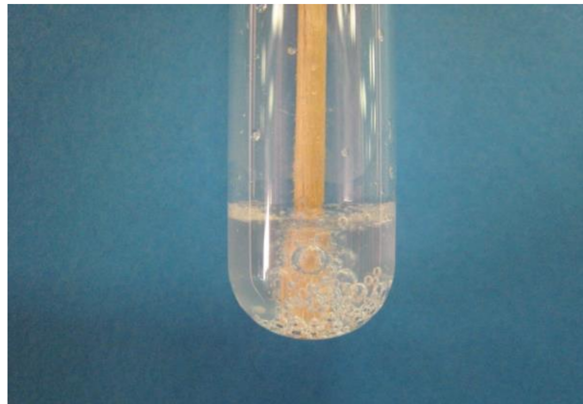

Figure 26: Positive catalase reactions obtained by using tube method.

In general, the test of catalase is used to detect whether or not an unknown bacterium has the enzyme, catalase. All members of the genus *Staphylococcus* have the catalase enzyme. The catalase test is the differential test between the genera *Staphylococcus* and *Streptococcus*.

3.4. Slide coagulase test procedure

The test of coagulase can be performed using two different procedures. The slide test is simple, giving results within 10 seconds, but it can give false negatives. The tube test is the definitive test, however, it can take up to 24 hours to complete. For both tests, clumping or clots of any size indicate a positive response (**Blair, 1939; Turner & Schwartz**, **1958**). Coagulase test is used to differentiate *S. aureus* (positive) from Coagulase Negative *Staphylococcus*. Coagulase

is an enzyme produced by *S. aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin. *S. aureus* produces two forms of coagulase, bound and free.

1. Slide coagulase test is done to detect bound coagulase or clumping factor (Clumping factor directly converts fibrinogen to fibrin causing agglutination).
2. Tube coagulase test is done to detect free coagulase.

3.4.1. Slide test

This test detects clumping factor (formerly referred as cell-bound coagulase).

1- Emulsify a staphylococcal colony in a drop of water on a clean and grease-free glass slide with a minimum of spreading (*If the isolate does not form a smooth, milky suspension, do not proceed with the test*).
2- Make similar suspensions of control positive and negative strains to confirm the proper reactivity of the plasma.
3- Dip a flamed and cooled straight inoculating wire into the undiluted plasma at room temperature, withdraw, and stir the adhering traces of plasma (not a loopful) into the staphylococcal suspension on the slide. Flame the wire and repeat for the control suspensions.
4- Read as positive a coarse clumping of cocci visible to the naked eye within 10 seconds. Read as negative the absence of clumping or any reaction taking more than 10 seconds to develop, but re-examine any slow reacting strains by the tube coagulase test.

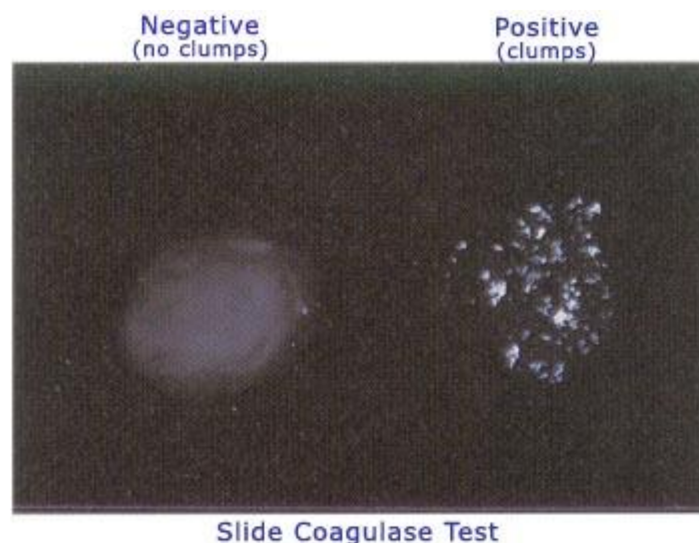

Figure 27: Slide coagulase test. Coagulase-negative staphylococci are present on the left side of the slide, while coagulase-positive staphylococci are present on the right side of the slide.

Slide coagulase test is the main method used to identify *S. aureus* in clinical laboratories but it has some limitations.

1. About 15% of ordinary strains of *S. aureus* and many more of MRSA give negative reactions.

2. Few species of coagulase-negative staphylococci give positive reactions.

Note: All coagulase negative slides must be confirmed using a tube coagulase test as the definitive test for *S. aureus*.

3.4.2. Tube coagulase test

1. Detects staphylocoagulase which reacts with coagulase-reacting factor (CRF). CRF is a thrombin-like molecule.

2. Staphylocoagulase and CRF combine to indirectly convert fibrinogen to fibrin.

3. A suspension of the organism is suspended and incubated with plasma at 37°C.

4. Clot formation within 4 hours indicates a positive test.
   - Positive test indicates *S. aureus*
   - Some species of Coagulase negative *staphylococcus* can be positive

5. Negative tubes should be held overnight at room temperature.
   - Some species possess enzyme that can cause the dissolution of clot after prolonged incubation.

3.4.2.1. Tube coagulase test procedure

- Prepare a 1-in-6 dilution of the plasma in saline (0.85% NaCl) and place 1 ml volumes of the diluted plasma in small tubes.
- Emulsify several isolated colonies of test organism in 1 ml of diluted rabbit plasma to give a milky suspension.
- Incubate the tube at 35°C in ambient air or in a water bath for 4 hours.
- Examine at 1, 2 and 4 hours for clot formation by tilting the tube through 90°. (Clots may liquefy after their formation).
- Leave negative tubes at room temperature overnight and re-examine. (*This step is essential, for some strains of S. aureus, including many MRSA, produce a delayed clot which is rapidly lysed at 37•C by the organism's staphylokinase*.)

Observation,

Read as positive any degree of clot formation. Often the plasma is converted into a stiff gel that remains in place when the tube is tilted or inverted, but sometimes clots are seen floating in the fluid.

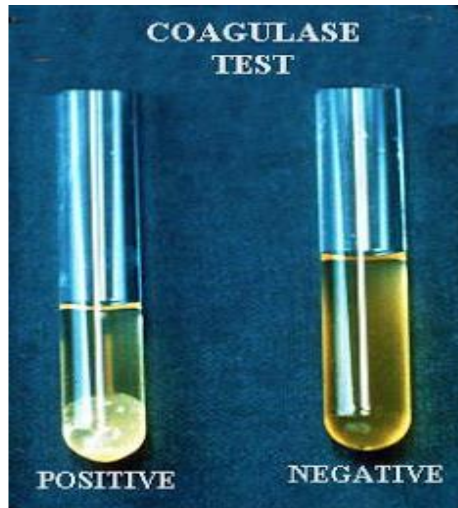

Figure 28: Coagulase test. **Coagulase Positive**: Clot of any size, for example, *S. aureus* are present on the left side of the slide, while **Coagulase Negative**: No clot (plasma remains wholly liquid or shows only a flocculent or ropy precipitate, for example, *S. epidermidis* are present on the right side of the slide.

Because of the presence of other species within coagulase positive *Staphylococcus* group such as *S. intermedius*, *S. pseudintermedius*, *S. delphini* could be led to false identification of *S. aureus* by test previously described above. For this reason, the need of more specific tests like Slidex Staph Plus and API galleries are often required in order to complete the identification of *S. aureus* from other coagulase positive *staphylococcus* group.

3.5. Slidex Staph Plus (Testing for Fibrinogen Receptor and protein A) testing

The role of this test is identification of *S. aureus*. This test allows the detection of constituents, specific to the species *S. aureus*, present on the surface of bacteria: fibrinogen receptor and / or protein A. It is based on an agglutination reaction between the staphylococci strain grown on agar and sensitized particles.

Sensitized particles are particles carrying the complementary structure of the desired component. In the presence of bacteria carrying this component, they form agglutinates visible to the naked eye.

The particles used may be red blood cells or sensitized latex particles:

- by fibrinogen for FR detection: **Staphyslide test**
- with IgG for detection of protein A: **Aurea kit**

- by both: **Pastorex staph**

Marketed kits usually allow simultaneous search for these two constituents. The new generations of kits allow, in addition, the search for a third factor: the particles carry monoclonal antibodies specific for envelope antigens, present in certain strains of *S. aureus*. Boxes marketed: Pastorex staph, Slidex staph kit . . . .

- Kit for simultaneous research of FR and protein A

Slide agglutination test feasible from colonies of staphylococci collected on selective medium or not.

Place on a perfectly clean blade or on a single-use card:

- 1 drop of test reagent consisting of sensitized particles: latex (or red blood cells);

- 1 drop of human reagent consisting of the same non-sensitized particles.

- Take 1 to 2 colonies to identify, put them carefully in suspension in each of the 2 drops.

- Swirl slowly.
- Check for agglutination with the control reagent.
- Observe the appearance of massive agglutination of the test particles in less than 30 seconds.

Observation,

A clear agglutination of the test particles, while the control suspension remains homogeneous indicates that the staphylococcus studied has the FR and / or the protein A, so that it belongs to the species *S. aureus* (**Figure 29**):

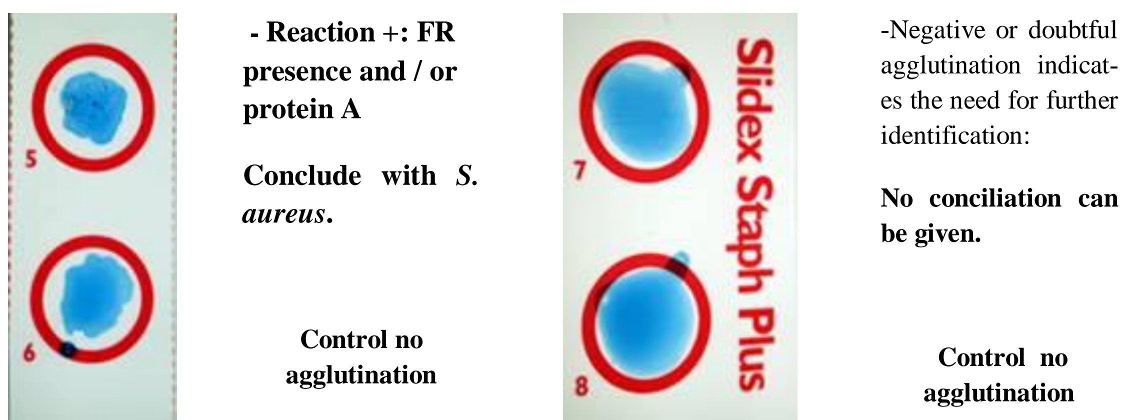

Figure 29: Different Slides Staph Plus reactions.

3.6. API® Staph

API® Staph is a standardized system for the identification of the genera *Staphylococcus*, *Micrococcus* and *Kocuria*, which uses miniaturized biochemical tests and a specially adapted database. The API galleries comprise 25 microtubes containing dehydrated substrates. Conventional tests are inoculated with a saline bacterial suspension that reconstitutes the media. The reactions produced during the incubation period result in spontaneous color changes or revealed by the addition of reagents.

The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software (**Figure 30, 31**).

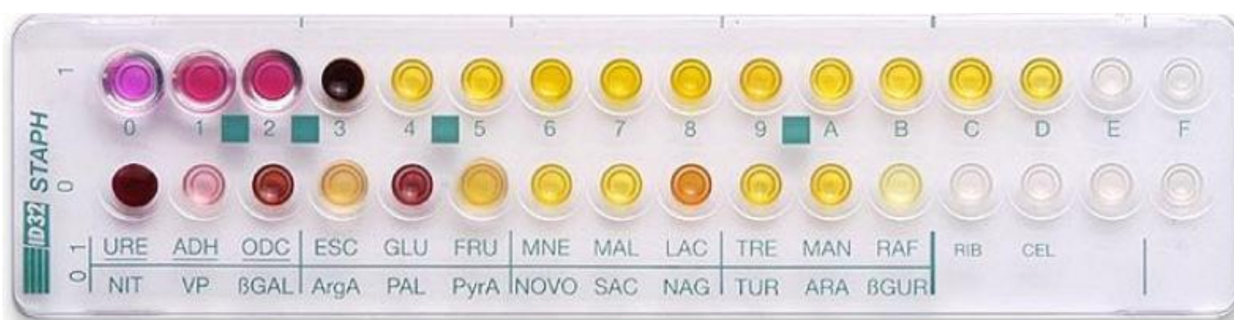

Figure 30: The API Staph galleries.

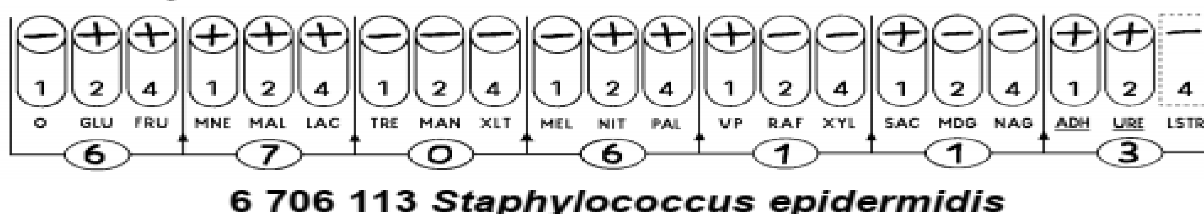

Figure 31: An example of how results are interpreted after period incubation of API galleries.

4. DNA extraction

For nucleic acid extraction from staphylococcal isolates, the samples will be firstly cultivated on Columbia ANC + 5% sheep blood (BioMérieux, Marcy l'étoile - France) and incubated immediately at 37C° for 24h up to 48h. After the incubation, 1-2 colonies growing on sheep blood and confirmed as *S. aureus* by using others tests described above, will be cultivated again in brain-heart infusion broth (Merck, Germany) at 37°C overnight. After this last incubation, genomic DNA was extracted from the samples using Phenol/Chloroforme/Isoamyl method or by using the QIAamp ^{R} DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer instructions (**Figures 32**).

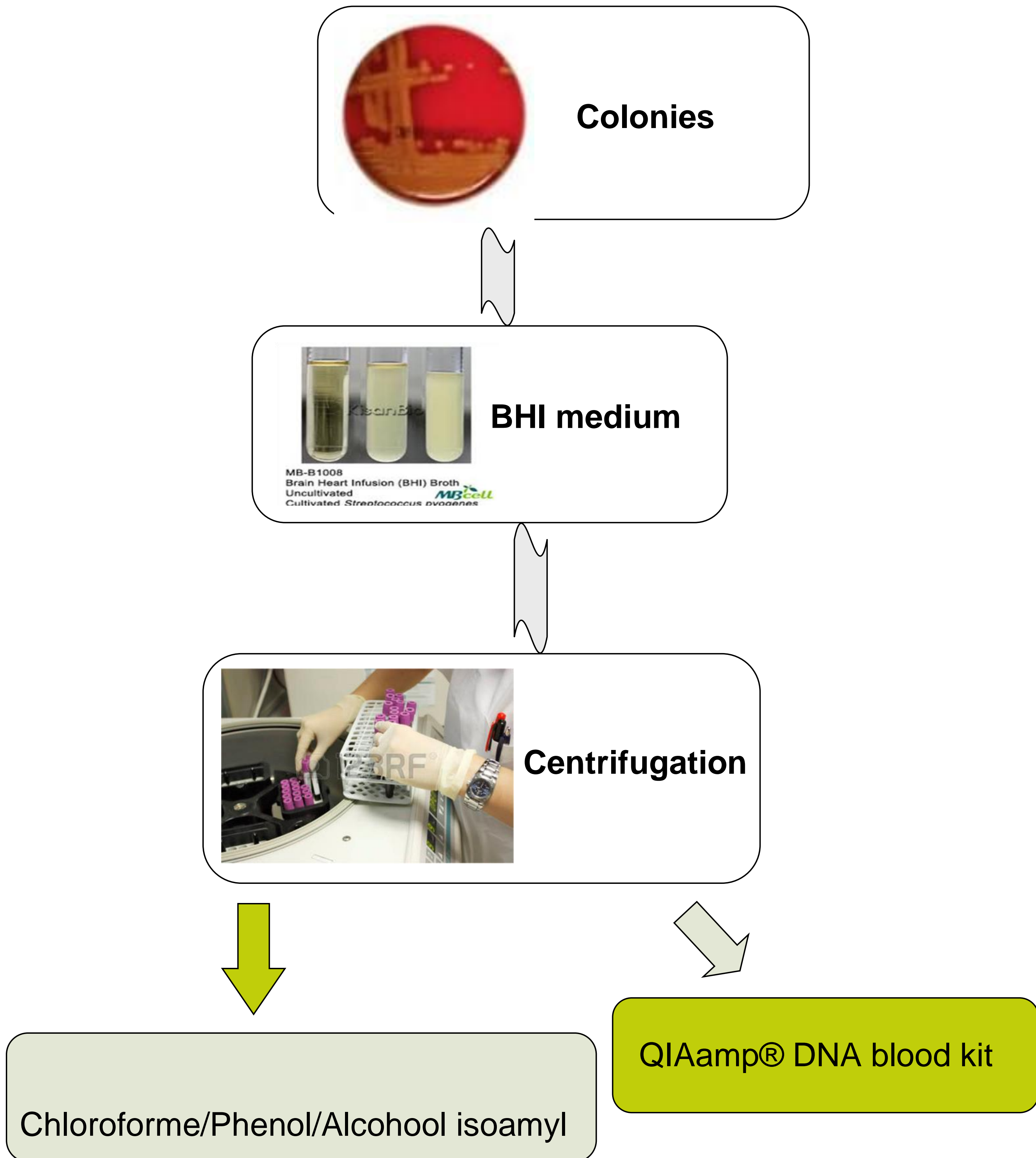

Figure 32: Representation of methods followed for DNA extraction.

4.1. DNA extraction by using Chloroforme/Phenol/Alcohool isoamyl method

1- Mix in micro centrifuge tube: - 166 µl of sampples
- 33 µl of 6Xpk Buffer
- 5 µl of PK (Proteinase K).

2- Incubate for 1 hour at 56°C in wather-bath.

3- Add an equal volume (200 µl) of Chloroforme/Phenol/Alcohool isoamyl method (25:24:1).

4- Vortex vigorously for 10 second.

5- Centrifuge at 14 000 rpm for 10 min in microcentrifuge. The DNA is in the upper, aqueous phase.

6- Remove the DNA carefully and transfer to a clean microcentrifuge tub. If a thick interphase is present, adjust the volume to 200 µl with ddH2O and repeat steps 2 and 3.

7- Extract with 200 µl of chloroforme and centrifuge as in step 3

8- Remove the DNA to a clean microcentrifuge tube and precipate by adding 2.5volume of ice-cold 95% - 100% ethanol and 0.1 volume of 3M NaAC, Ph 5.2.

9- Mix by flicking the tubes.

10- Place at – 20°C for at least 30 min (preferably overnight).

11- Centrifuge again at 14 000 rpm for 30 min to pellet DNA.

12- Wash the pellet with 1 mol of 70% ethanol.

13- Remove the ethanol without distrubting the pellet and air-dry the pellet.

14. Resuspend the pellet in 20-50 µl of ddH2O. Finally, DNA can then be used for further processing or stored at – 20°C until used.

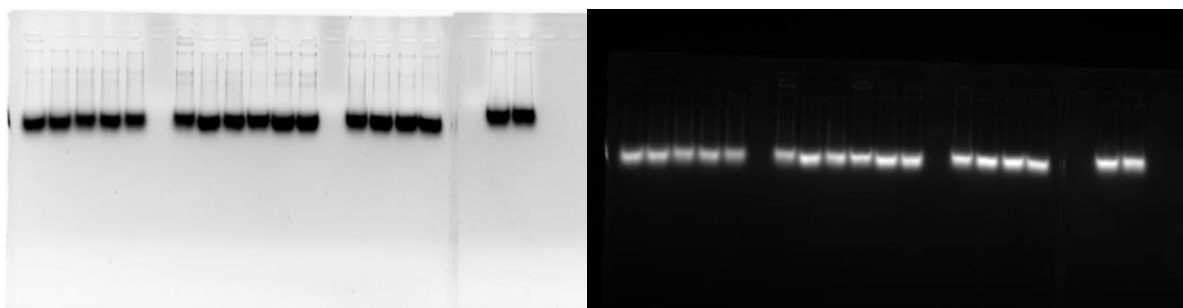

Figure 33: Example of DNA fragment of *S. aureus* isolates visualised under UV light and photographed on a Gel DocTM XR Imaging System (Bio-Rad, California, USA) by using Chloroforme/Phenol/Alcohool isoamyl method.

4.2. DNA extraction by using QIAamp® DNA blood kit method

DNA extraction from *S. aureus* strains incubated on Blood agar media during 24h has also been carried out using QIAamp® DNA blood kit method according to the manifucture's instructions (**QIAamp® DNA Mini and Blood Mini Handbook, 2016**).

4.2.1. Isolation of genomic DNA from Gram-positive bacteria using kit method

1. Pellet bacteria by centrifugation for 10 min at 5000 x g (7500 rpm).

2. Suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/ml lysozyme or 200 µg/ml lysostaphin; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).
3. Incubate for at least 30 min at 37°C.

4. Add 20 µl proteinase K and 200 µl Buffer AL. Mix by vortexing.
5. Incubate at 56°C for 30 min and then for a further 15 min at 95°C.

Note: Extended incubation at 95°C can lead to some DNA degradation.

6. Centrifuge for a few seconds.

7. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

8. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. *

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach.

Close each spin column to avoid aerosol formation during centrifugation. It is essential to apply all of the precipitate to the QIAamp Mini spin column.

Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate. *

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

10. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

11. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

12. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

13. Repeat step 12.

A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield. Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

For long-term storage of DNA, eluting in Buffer AE and placing at –30 to –15°C is recommended, since DNA stored in water is subject to acid hydrolysis.

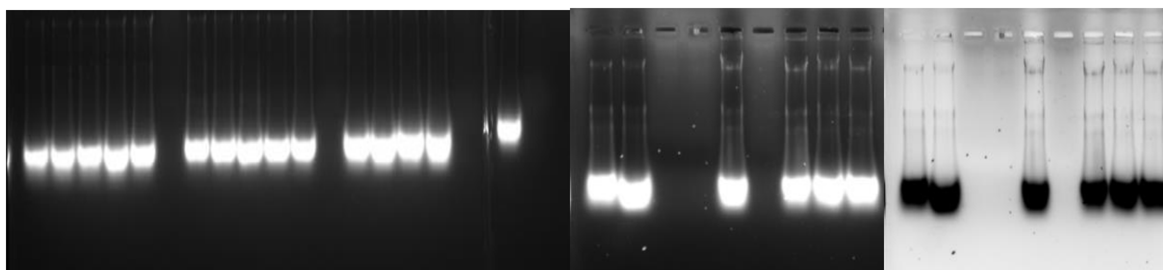

Figure 34: Example of DNA fragments of *S. aureus* isolates visualised under UV light and photographed on a Gel DocTM XR Imaging System (Bio-Rad, California, USA) by using QIAamp® DNA blood kit method.

5. Primers used for PCR

Primers exploited and used in this present study were synthesized and also purchased from Invitrogen Company (Invitrogen^{TM} , Paisley, UK). All isolates were tested using the primer pairs shown in **Table 8**.

Table 8: Primers used for the detection of antibiotic resistance genes by PCR.

Antibiotics	Genes	Primers sequences (5` - 3`)	Amplicons size
Beta-lactams	*BlaZ*	487 fw TAAGAGATTTGCCTATGCTT 373 fw TTAAAGTCTTACCGAAAGCAG	377 bp
Beta-lactams	*MecA*	MecA fw AAAATCGATGGTAAAGGTTGG MecA rev AGTTCTGCAGTACCGGATTTGC	533 bp
Glycopeptides	*Sal(A)*	salA fw CGATGAACCAACAAACCACA salA rev AGGACCGAACCTTGAAATGA	931 bp
Tetracyclines	*tet(K)*	tetK fw GTAGCGACAATAGGTAATAGT tetK rev GTAGTGACAATAAACCTCCTA	360 bp
Tetracyclines	*tet(M)*	tetM fw AGTGGAGCGATTACAGAA tetM rev CATATGTCCTGGCGTGTCTA	158 bp
Aminosides	*aac(6`)-aph (2``)*	Aac fw GAAGTACGCAGAAGAGA Aac rev ACATGGCAAGCTCTAGGA	491 bp
Aminosides	*Aph(3`)-IIIa*	aph fw AAATACCGCTGCGTA aph rev CATACTCTTCCGAGCAA	242 bp
Aminosides	*Ant(4`)-Ia*	ant fw AATCGGTAGAAGCCCAA ant rev GCACCTGCCATTGCTA	135 bp
Aminosides	*Spc*	Spc fw ACCAAATCAAGCGATTCAAA Spc rv GTCACTGTTTGCCACATTCG	561bp

Continued Table.

Lincosamides	*Vga(C)*	vgaC fw CCGTATGCCCAGAGTGAGAT vgaC rev TGCTTGGGAACAAGTCCTTC	671 bp
Lipopeptides	*DfrK*	dfrK fw GCTGCGATGGATAATGAACAG dfrK rev GGACGATTTCACAACCATTAAAGC	214 bp
Macrolides	*Erm(T)*	ermT fw ATTGGTTCAGGGAAAGGTCA ermT rev GCTTGATAAAATTGGTTTTTGGA	536 bp
Glycopeptides	*Van(A)*	vanA fw ATGAATAGAATAAAAGTTGC vanA rev CACCCCTTTAACGCTAATA	1032 bp
Lincosamide	*Lnu(C)*	lnuC fw AATTTGCAATAGATGCGGAGA lnuC rev TCATGTGCATTTTCATCA	1100 bp

6. PCR for antibiotic resistance genes

The Fifty-two *S. aureus* isolates were tested by PCR for the *blaZ*, *mecA, aac(6`)/aph(2``)*, *aph(3)-IIIa*, *ant(4``)-Ia*, *Spc*, *tet(M)*, *tet(K), erm(T), van(A), sal(A), lnu(C), dfrK and vga(C)* antibiotic resistance genes detection as previously described. Briefly, after DNA extraction all isolates were tested for the selected genes by PCR, using a respective set of specific primers (**Table 8**) in a thermocycler C 1,000 (Bio-Rad, California, USA) (**Figure 35**). The amplicons (**Table 8**) for the tested genes were visualized on a 1.5% (w/v) agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Duren, Germany) and visualized under UV light (Bio-Rad, California, USA).

Figure 35: Thermocycler C 1,000 apparatus.

6.1. Molecular detection of *blaZ* resistance gene by PCR method

PCR method was performed in a final volume of 25µl, containing 0.5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.15 µl of dNTPs (1mM), 0.15 µl of each *blaZ*-fw and *blaZ*-rev primers (10 Pmol) (**Table 8**), 0.5 µl of Taq DNA polymerase (5u/µl) and 16.5 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 3 min; 30 cycles of 95 °C for 30 S, 54 °C for 30 S, and 72 °C for 30 S, and a final extension step at 72 °C for 4 min (**Figure 37**).

6.2. Molecular detection of *mecA* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 0.5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 1 µl of dNTPs (1mM), 0.5 µl of each *mecA*-fw and *mecA*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.3 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 94 °C for 4 min; 35 cycles of 94 °C for 30S, 55 °C for 30S, and 72 °C for 30S, and a final extension step at 72 °C for 5 min (**Figure 37**).

6.3. Molecular detection of *aph(3)-IIIa* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *aph(3)-IIIa*-fw and *aph(3)-IIIa*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 2 min, 54 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min (**Figure 37**).

6.4. Molecular detection of *ant(4``)-Ia* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *ant(4``)-Ia*-fw and *ant(4``)-Ia*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle

conditions: an initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 2 min, 54 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min (**Figure 37**).

6.5. Molecular detection of *aac(6`)/aph(2``)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *aac(6`)/aph(2``)*-fw and *aac(6`)/aph(2``)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 2 min, 54 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min (**Figure 37**).

6.6. Molecular detection of *tet(M)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.15 µl of each *tet(M)*-fw and *tet(M)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 16.3 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 3 min; 40 cycles of 95 °C for 30S, 72 °C for 30S, and 72 °C for 30S, and a final extension step at 72 °C for 4 min (**Figure 37**).

6.7. Molecular detection of *tet(K)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.15 µl of each *tet(K)*-fw and *tet(K)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 16.3 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 3 min; 40 cycles of 95 °C for 30S, 72 °C for 30S, and 72 °C for 30S, and a final extension step at 72 °C for 4 min (**Figure 37**).

6.8. Molecular detection of *erm(T)* gene by PCR method

The PCR solution was performed in a final volume of 25 µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of

each *erm(T)*-fw and *erm(T)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl), 0.75 µl of Mgcl2 and 14.8 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 3 min, and a final extension step at 72 °C for 3 min (**Figure 37**).

6.9. Molecular detection of *van(A)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.5 µl of each *van(A)*-fw and *van(A)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.8 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 5 min; 35 cycles of 95 °C for 2 min, 50 °C for 2 min, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min (**Figure 37**).

6.10. Molecular detection of sal(A) gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *sal(A)*-fw and *sal(A)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 3 min; 35 cycles of 95 °C for 30S, 57 °C for 30S, and 72 °C for 2 min, and a final extension step at 72 °C for 5 min (**Figure 37**).

6.11. Molecular detection of *lnu(C)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *lnu(C)*-fw and *lnu(C)*-rev primers primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 95 °C for 3 min; 35 cycles of 95 °C for 30S, 50 °C for 30S, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min (**Figure 37**).

6.12. Molecular detection of *dfrK* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.2 µl of each *dfrk*-fw and *dfrk*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 16.1 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, and a final extension step at 72 °C for 7 min (**Figure 37**).

6.13. Molecular detection of *vga(C)* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.2 µl of each *vga(C)*-fw and *vga(C)*-rev primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 16.1 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 3 min, and a final extension step at 72 °C for 7 min (**Figure 37**).

6.14. Molecular detection of *spc* gene by PCR method

The PCR solution was performed in a final volume of 25µl, containing 5 µl of template DNA prepared as described above, 2.5 µl of PCR buffer (10x), 0.5 µl of dNTPs (1mM), 0.625 µl of each *spc)*-fw and *spc*-rev primers primers (10 Pmol) (**Table 8**), 0.2 µl of Taq DNA polymerase (5u/µl) and 15.55 µl of double distilled water (**Figure 36**). PCR amplifications were performed with C1000^{TM} thermal cycler (Bio-Rad, California, USA), using the following cycle conditions: an initial denaturation step at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min (**Figure 37**).

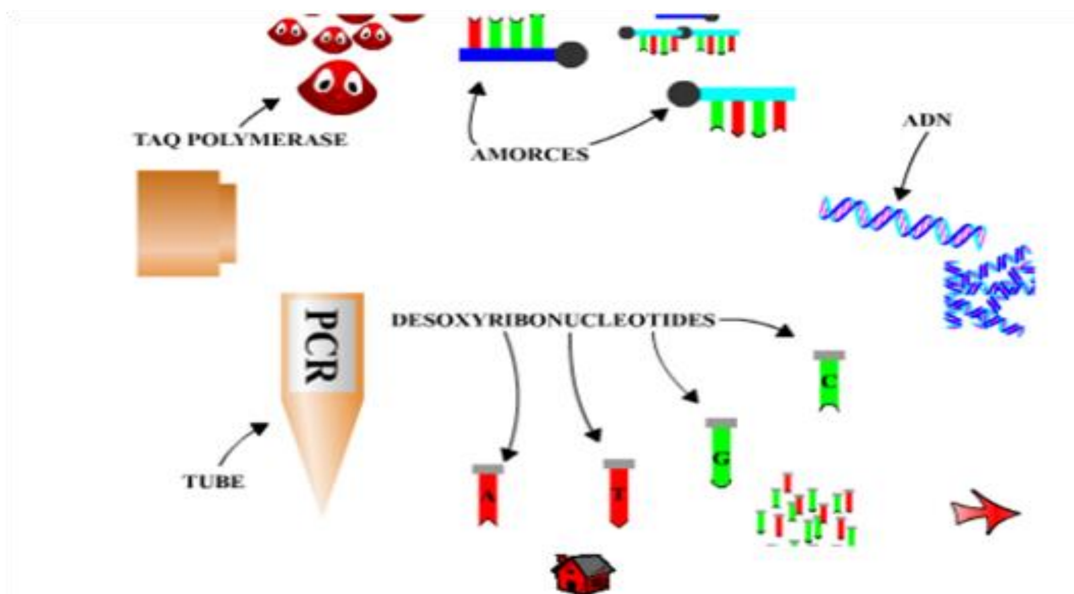

Figure 36: Essential constituants of PCR reactions.

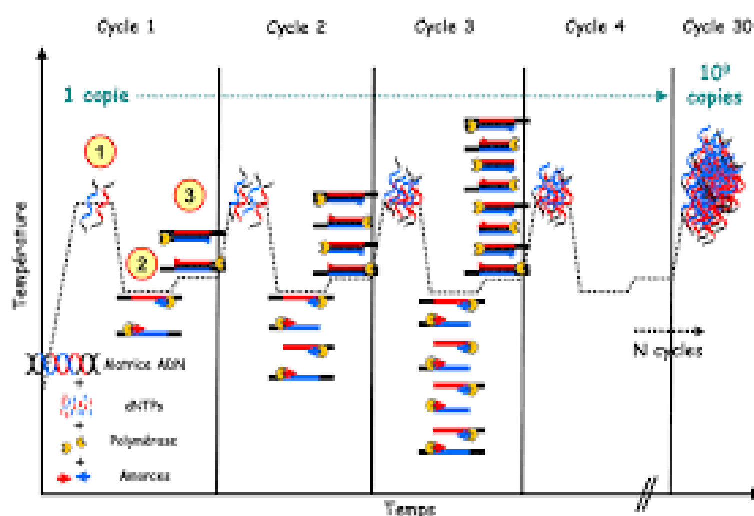

Figure 37: PCR amplifications cycle conditions.

7. Agarose gel electrophoresis

The amplified products were separated on 1.5 % agarose gel in 0.5 × TBE buffer (pH 8.3; 0.09 M Tris, 0.89 M boric acid, 20 mM Na2EDTA). The gels were stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH; Duren; Germany) to see the amplified DNA fragments (blaZ: 173bp; under UV light and photographed on a Gel Doc^{TM} XR Imaging System (Bio-Rad, California, USA) by comparison with a molecular size marker (100 bp ladders, GRS Ladder 100BP from grisp (Porto, Portugal)) was used as a DNA marker. The PCR

products were mixed with a sample loading buffer. Gels were run in 0.5 x TBE buffer at 100 V for X h. **Figure 38** show principal electrophoresis equipment.

Figure 38: Electrophoresis equipment.

8. Protocol for PCR clean-up or DNA purification from enzymatic reactions

1. Transfer the volume of the reaction mixture into a 1.5 ml microcentrifuge tube and add five volume of Binding Buffer. Mix by inverting the tube a few times. Centrifuge briefly to collect the sample. All purifications steps including centrifugation should be carried out at room temperature.
2. Add the above mixture to the NZYTech spin column and let stand for 2 minutes. The maximum loading volume of the column is 700 µl for sample volumes greater than 700 µl simply load again. Centrifuge for 1 minutes and discard the flow-through in the tube.
3. Add 600 µl of Wash and centrifuge for 1 minute. Discard the flow-through in the collection tube.
4. Centrifuge for 1 minute to dry NZYTech spin membrane of residual ethanol.
5. Place the NZYTech spin column into a clean 1.5 ml microcentrifuge tube. Add 50 µl of Elution Buffer to the centre of the column and incubate at room temperature for 1 minute. Centrifuge for 1 minute to elute DNA. Ultrapure water may be used in place of elution buffer. However, DNA recovery with acidic waters may be significantly reduced.

Note: *it is extremely important to add the Elution Buffer to the centre of the column. Incubating the column at higher temperature (30°C to 50°C) may slightly increase the yield. Pre-warming the Elution Buffer at 55 to 80°C may also slightly increase elution efficiency.*

9. Phylogenetic analysis

The generated partial amino acid sequences of *blaZ* gene were aligned using Clustal W through MEGA version 5.0 (Kimura, 1980) for phylogenetic inference. An evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the JTT matrix-based model

(**Jones *et al.*, 1992**). The tree with the highest log likelihood (-781.1857) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 200.0000). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 61 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0 (**Kimura, 1980**).

For the phylogenetic analysis, the consensus sequences were aligned with human, bovine and animal food *S. aureus* sequences, animal *S. warneri* and *S. intermedius* sequences, and with two *S. haemolyticus* sequences, one from air and the other from human/animal origin that was used as an outgroup. For all selected strains, respective Genbank accession no. is referred in the phylogenetic tree. **Figure 39** show different step of the phylogenetic analysis.

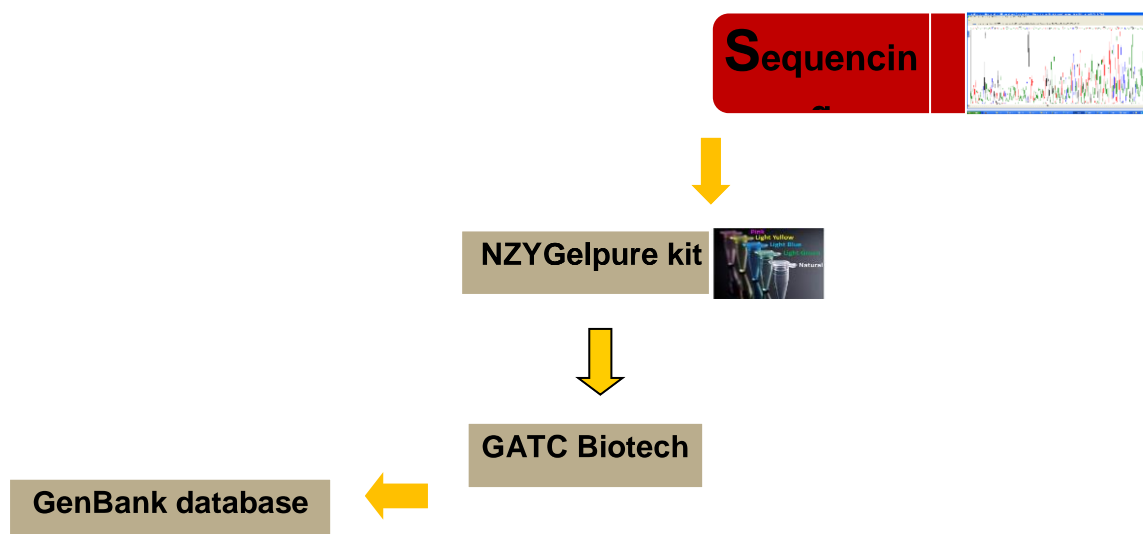

Figure 39: Different step of the phylogenetic analysis.

10. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed in all *S. aureus* isolates by the disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines (**CLSI, 2007; CLSI, 2014**) (**Figure 40**). Isolates streaked on Columbia ANC agar supplemented with 5% of sheep blood (bioMérieux, Marcy l'Etoile, France) were grown overnight at 37ºC. After, colonies were re-suspended in 1 ml of 0.85% (w/v) sodium chloride (Merck Laboratories, Darmstadt, Germany) and adjusted to 0.5 McFarland in comparison with a McFarland standard (bioMérieux, Marcy l'Etoile, France). Then, Mueller–Hinton agar (Merck

Laboratories, Darmstadt, Germany) plates were inoculated with the inoculum by dipping sterile cotton swabs into the bacterial suspension. The antibiotics groups tested were from:

- Family of beta-lactams,

The chosen antibiotics were as follows : Group of Penicillins G (penicillin (6ug) (PEN)); group of Penicillins M (oxacillin 1ug (OXA)), group of Aminopenicillins (ampecillin (10ug) (AMP), amoxicillin (25ug) (AM), amoxicillin + clavulanic acid (20ug+10ug)) (AM+CA); group of Carboxypenicillins (ticarcillin (75ug) (TIC)); group of Ureidopenicillins (piperacillin (100ug) (PIP)); group of Carbapenems (imipenem (10ug) (IMP)); group of Monobactames (aztreonam (30ug) (AZT)) and group of Cephalosporines (cefazolin (30ug) (CFZ)).

- Family of Aminoglycosides,

The chosen antibiotics were as follows: gentamicin (GMN 10μg), neomycin (NMN; 30μg), tobramycin (TMN 10μg), amikacin (AKN 30μg), streptomycin (STN 10μg) and kanamycin (KMN 30μg).

- Family of Tetracyclines,

The chosen antibiotic was tetracycline (TET 30μg).

- Family of Macrolides,

The chosen antibiotics were as follows: erythromycin (ERY 15μg) and clindamycin (CLDM 2μg).

- Family of Glycopeptides,

The chosen antibiotic was vancomycin (VANCO, 30μg).

Other antibiotics class such as chloramphenicol (30μg) (CL), trimethoprim/sulfamethoxazole (1,25μg+23,75μg) (TMP/SMX), novobiocyn (NOVO) and polymixine (50μg) (PLX) (**Lipopeptides family**) was also shosen for susceptibility testing.

All antibiotics used in this study were purchased from BioMerieux (BioMérieux, Marcy l'étoile – France) and from Oxoid (Oxoid, Thermo Fisher Scientific Inc., Lenexa, KS, U.S.A). The inoculated agar plates with discs were incubated for 24 h at 37°C. Following the incubation, the diameters of the inhibition zones were measured in millimeters and compared with the ranges suggested by the CLSI guidelines or only on the basis of the inhibition zones. The strains were classified as resistant, intermediate or susceptible on the basis of the size of the inhibition zone.

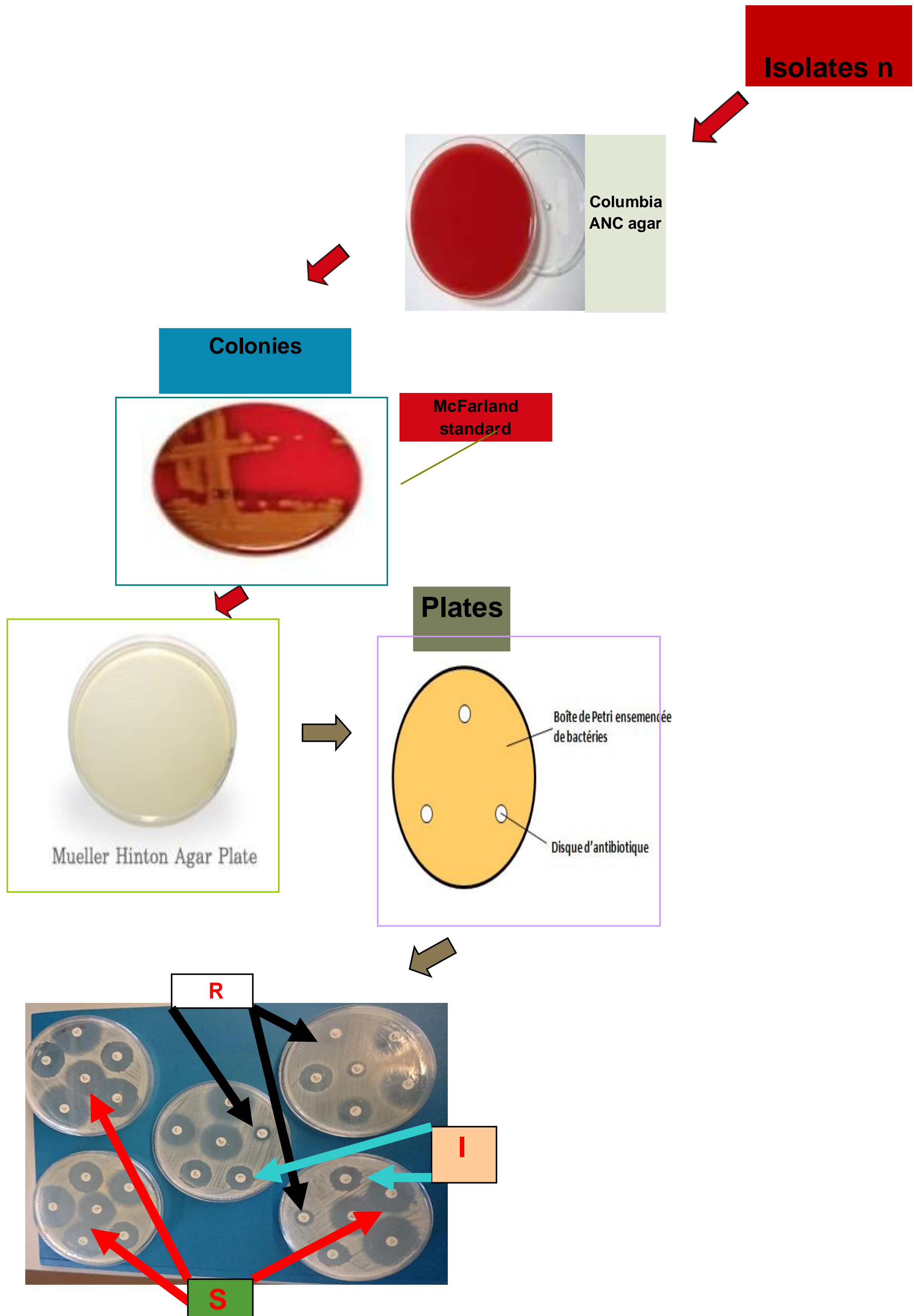

Figure 40: The antimicrobial susceptibility testing method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (**CLSI, 2007; CLSI, 2014**).

Results and Discussion

Antimicrobial resistance is a phenomenon that occurs naturally over time, usually through genetic changes. However, the misuse and overuse of antimicrobials is accelerating this process. In many parts of the world, antibiotics are overused and misused in people and animals, and often given without professional oversight (**WHO, 2018**). Nowadays, drug-resistant microorganism is a growing global danger. Strains of *S. aureus* have developed resistance to many commonly used antimicrobials due to indiscriminate use of antimicrobials, and treatment becoming a challenge (**Deyno *et al.,* 2017**). The continuing evolution of antimicrobial resistance in the world forces the scientists to work in this field permantly. Continuous surveillance of antibiotic resistance, especially that caused by very dangerous pathogenic germs, such as *S. aureus*, is important for the knowledge and understanding of its evolution in the Portugal, by monitoring the resistance to the antibiotics which are used to fight or prevent infections caused by *S. aureus* especially in case of mastitis disease in Portugal and to follow the genes of the most widespread and circulating resistances which can contribute to the implementation of more adequate control measures and effective of the mastitis disease. Therefore, the main objectives of this thesis went in this direction to have an image on the phenotypic and genotypic resistance profiles of *S. aureus* to the different families of antibiotics such as Beta-lactams, Aminoglycosides, Glycopeptides, Peptides, Tetracyclines and Macrolides. The specific aims of this work were:

Part I:

- Investigate the susceptibility of a set of antibiotics representing all groups of the Beta-lactams family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, in years 2003-2004, 2007-2008 and 2017. Moreover, the detection by specific PCR methods of the *blaZ* and *mecA* resistance genes was also evaluated as well as the phylogenetic analysis of partial *blaZ* gene consensus sequences in selected isolates.

Part II:

- Evaluate the efficacy of a set of antimicrobials from different antibiotics family such as Aminosides family; Tetracyclines family; Macrolides family; Glycopeptides family and Lipopeptides family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, in years 2003-2004, 2007-2008 and 2017. Moreover, the detection by specific PCR methods of the *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *tet(M)*, *tet(K), erm(T), van(A), sal(A), lnu(C), dfrK* and *vga(C)* antibiotic resistance genes was also evaluated in selected isolates.

Part I:

Efficacy of Beta Lactams Antmicrobials: Case of *Staphylococcus aureus* Resistance Isolated from Mastitis Bovine in Northwest of Portugal.

Abstract

***Objectives*:** Investigate the antimicrobial susceptibility of twenty-six *S. aureus* bovine mastitis isolates obtained from 22 dairy herds from the northwest of Portugal against antibiotics belonging to the β-lactam family; detect and study the *blaZ* and *mecA* resistance genes diversity in positive isolates.

***Methods*:** The antimicrobial susceptibility tests were performed by the disk diffusion method. The detection of *blaZ* and *mecA* was performed using specific PCRs and the diversity of *blaZ* was evaluated by phylogenetic analysis.

***Results*:** The antimicrobial susceptibility test showed a prevalence of phenotypic resistance by *S. aureus* of 76.9% against penicillin, 73.0% against ampicillin and 3.8% against oxacillin and amoxicillin. A 100.0% phenotypic susceptibility was found against amoxicillin plus clavulanic acid, cefazolin, piperacillin, imipenem and ticarcillin. All tested isolates were PCR positive for *blaZ* and only isolate 25 was positive for *mecA*. The phylogenetic analysis of the detected *blaZ* placed the isolates in 3 different clusters that are closely related to other different bovine mastitis and human *S. aureus* strains.

Conclusion: A high prevalence of phenotypic resistance was found in the tested *S. aureus* isolates against penicillin and ampicillin. A prevalence of 100.0% was found for *blaZ* resistance gene. The phylogenetic analysis placed the isolates within 3 clusters closely related to different bovine mastitis and human *S. aureus* strains, with isolate 2 being the most divergent. Within clusters 1 and 3, most isolates are related with high prevalences of penicillin and ampicillin resistance. The isolates with a phenotypic susceptibility of 100.0% are included in cluster/subcluster 1 and cluster 2.

Introduction

S. aureus is a Gram positive bacteria (**Rybak *et al.,* 2000**) considered as an important human pathogen and known as one of the most important agents causing disease related with bovine mastitis in the world (**Olsen *et al.,* 2006**). Staphylococcal mastitis is considered as a major problem of the dairy industry in several countries (**Barkema *et al.,* 2006**). Mastitis, important disease encountered in dairy herds, consists of an inflammation of the mammary gland, usually developed in response to intramammary bacterial infection. They are the most common and most costly disease in dairy farming encountered (**Seegers *et al.,* 2003**). Several microorganisms, about 140 species, have been recognized as etiological agents of bovine mastitis (**Watts, 1988**), coliforms, streptococci and staphylococci are most often isolated (**Tenhagen *et al.,* 2006; Piepers *et al.,* 2007; Malinowski & Kłossowska, 2010; Smulski *et al.,* 2010;**). *S. aureus* is of particular importance, because it is highly infectious (**Kerro *et al.,* 2002**) and it is characterized by significantly lower cure levels in comparison with infections caused by other microorganisms (**Cramton *et al.,* 1999**). Moreover, the *S. aureus* has the potential to expand resistance to almost all the antimicrobial agents (**Barkema *et al.,* 2009; Hiramatsu *et al.,* 2001**). Beta-lactam antibiotics compounds such as penicillin maintains to be one of the mainly frequently addressed drugs in veterinary medicine (**Pitkala *et al.,* 2007**).

In the world, antimicrobial resistance of *S. aureus* is extensively spreading accompanied with extensive utilization of antibacterial agents in bovine mastitis. This antimicrobial resistance phenomenon is developed by the pathogens, and this could represent one of main reasons of low cure rate of mastitis (**Barkema *et al.,* 2006; Gao *et al.,* 2012**). Alternatively, this low rate could be also attributed to the unusually requent acquisition of antibiotic resistance mechanisms among this group of bacteria and also their ability to form biofilm (slime) (**Cramton *et al.,* 1999**). Of interest, the resistance phenomenon developped by *S. aureus* to beta-lactam antibiotics is complicated but primarily associated to the *blaZ* (**Olsen *et al.,* 2006**) and *mecA* genes (**Hartman & Tomasz, 1984**). *BlaZ* is the gene encoded for beta-lactamase enzyme that destroys susceptible beta-lactam antibiotics while *mecA* is the gene encoded for penicillin-binding protein 2a (PBP2a), which is not well inhibited by beta-lactams, making cell wall cross-linking possible of bacteria despite the presence of antibiotics (**Cha *et al.,* 2007**). These both genes are regulated by beta-lactam sensor/signal transducer proteins namely *BlaR1* and *MecR1* and repressor genes *blaI* and *mecI* (**Cha *et al.,* 2007**). The detection of the presence of *blaZ* has been well discovered in case of staphylococci from human and cattle origin (**Olsen *et al.,* 2006; Asfour *et al.,* 2011**) as well as in case of dogs and cats (**Malik *et al.,* 2007**).

In animal farms, mastitis bovine stays also one of the most frequent diseases in dairy sector cows. It is recognized also as an endemic disease and considered to be the most prevalent and mainly expensive disease touching and confronting the dairy farm (**Miller *et al.,* 1993; Gruet *et al.,* 2001; Halasa *et al.,* 2007**). Economic losses caused by mastitis represent lower revenues and higher costs on milk production as compared with healthy cows. Despite considerable researches that have been done on bovine mastitis, the disease still remains an economically relevant problem to the dairy industry (**DeGraves *et al.,* 1993**). The evident economic impact of bovine mastitis has taken a place of discussion by a various papers.

Thus, to simplify their conclusions, economic losses caused by clinical or subclinical mastitis in dairy farms are due to the following factors: Observed production losses, use of drugs, discarded milk, labour, materials and investments, veterinary services, diagnostics, risk of other diseases and culling. The relative costs of these factors described might differ between countries and also between regions, but it appears that the economic principles behind them are the same (**Gruet *et al.,* 2001; Halasa *et al.,* 2007**).

In 1970, total annual economic losses in the United States only attributable to mastitis have been estimated to be between $400 to $500 million (**Janzen, 1970**). In Dutch circumstances, the average costs of a case of clinical mastitis bovine are estimated at €277 and €168 for cows in early and late lactation respectively whereas in UK circumstances the average costs are estimated at £203 (**Hogeveen, 2005**). The economic loss per one case of mastitis occurrence was estimated in Czech Republic at 9000 CzC [around 360 €] / (1 €= 25 CzC (Czech Crown)). The losses caused by mastitis can vary between 4000 and 18 000 CzC according to various factors that involved, for example the occurrence and intensity of disease (**Kvapilík *et al.,* 2015**). Based upon a data available on-line, the Canadian Bovine Mastitis and Milk Quality Research Network (**CBMQRN, 2016**) evaluate that in Canada the total loss associated with this important disease to more than $400 million per year.

The cattle sector in the world provides a means to existence of a greater number of people than any other industry. Although the use of veterinary drugs by veterinarians are sometimes imperative and plays a major role in the control of diseases in cattle populations, good management and preventive practices in the herds can help the reduction of disease expression affecting this vital sector and consequently the need to resort to drugs that should be done wisely.

In order to gather a set of information on Beta-lactam family concerning the resistance of *S. aureus* from bovine mastitis against this antimicrobial family; the first objective of our study was to test the susceptibility of a set of antibiotics representing all groups of the Beta-lactam

family by using the disk diffusion method, against 26 *S. aureus* isolates from bovine mastitis collected from 22 different dairy herds from the northwest of Portugal in years 2003-2004 and 2007-2008. Moreover, our second objective was to detect by using specific PCR methods the presence of the *blaZ* and *mecA* resistance genes that was also evaluated as well as the phylogenetic analysis of the *blaZ* in the same isolates.

Bacterial strains

A series of 26 Staphylococcus isolates isolated from milk samples received from clinical and subclinical mastitis bovine at the laboratory of Infectious Diseases of the Veterinary Clinics Department at Institute of Sciences Biomedical Abel Salazar (ICBAS), University of Porto (Portugal) since year 2003 to 2008 were used and included in this study in order to maintain and force the scientific connection between farms and research laboratory. The availability of strains has originated from 22 farms situated in North of Portugal [farm 1 (3 isolates), farm 2 (1 isolate), farm 3 (1 isolate), farm 4 (1 isolate), farm 5 (1 isolate), farm 6 (1 isolate), farm 7 (1 isolate), farm 8 (2 isolates), farm 9 (1 isolate), farm 10 (1 isolate), farm 11 (1 isolates), farm 12 (1 isolate), farm 13 (1 isolate), farm 14 (1 isolate), farm 15 (1 isolate), farm 16 (1 isolate), farm 17 (1 isolate), farm 18 (1 isolate), farm 19 (1 isolate), farm 20 (2 isolates), farm 21 (1 isolate), farm 22 (1 isolate)] (**Table 9**).

Table 9: Herds and year of collection of *S. aureus* isolates.

Farm	Isolates	Year
1	1 / 2 and 3	2003 / 2008
2	4	2003
3	5	2004
4	6	2003
5	7	2003
6	8	2003
7	9	2004
8	10 and 11	2003
9	12	2003
10	13	2004
11	14	2003
12	15	2003
13	16	2003
14	17	2004
15	18	2003
16	19	2004
17	20	2007
18	21	2008
19	22	2008
20	23 and 24	2008
21	25	2008
22	26	2008

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed with equivalence of 0.5 McFarland turbidity standards by agar disc diffusion method on Mueller-Hinton agar plates following the guidelines of Clinical and Laboratory Standards Institute (**CLSI, 2007; CLSI, 2014**). The antibiotics groups tested were from family Beta-lactams, the chosen antibiotics were as follows: Group of Penicillins G (penicillin (6ug)); group of Penicillins M (oxacillin 1ug), group of Aminopenicillins (ampicillin (10ug), amoxicillin (25ug), amoxicillin + clavulanic acid (20ug+10ug)); group of Carboxypenicillins (ticarcillin (75ug)); group of Ureidopenicillins (piperacillin (100ug)); group of Carbapenems (imipenem (10ug)); group of Monobactames (aztreonam (30ug)) and group of Cephalosporines (cefazolin (30ug)). All antibiotics used in this study were purchased from BioMerieux (BioMérieux, Marcy l'étoile - France). The inoculated agar plates with discs were incubated for 24 h at 37°C. Following the incubation, the diameters of the inhibition zones were measured in millimeters and compared with the ranges suggested by the CLSI guidelines. The strains were classified as resistant, intermediate or susceptible on the basis of the size of the inhibition zone.

PCRs of *blaZ* and *mecA* resistance genes and sequencing

PCRs targeting *blaZ* and *mecA* genes were performed accordantly as previously described (**Olsen *et al.,* 2006; Szweda *et al.,* 2014**). Briefly, after DNA extraction all isolates were tested for *blaZ and mecA* using the set of primers 487 (5′-TAAGAGATTTGCCTATGCTT-3`)/373 (5′-TTAAAGTCTTACCGAAAGCAG-3`) and *mecA*fw (5′-AAAATCGATGGTAAAGGTTG G-3`)/ *mecA*rev (5′-AGTTCTGCAGTACCGGATTTGC-3`) respectively, by PCR in a thermocycler C 1,000 (Bio-Rad, California, USA). The amplified products (*blaZ*-377bp and *mecA*-533bp) were analyzed on a 1.5% (w/v) agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Duren, Germany) and visualized under ultraviolet light (Bio-Rad, California, USA). Following the amplification, 26 *blaZ* gene amplicons, selected for sequencing, were purified with the NZYGelpure kit (nzytech, Lisbon, Portugal) and directly sequenced at GATC Biotech (Cologne, Germany) using the same primers. Retrieved sequences were analyzed and a consensus sequence for each isolate was created after overlapping of the obtained sequences from forward and reverse primers using MEGA version 5.0 (**Kimura, 1980**). All sequences were deposited in GenBank database under accession nos. KY020052 (isolate 1,herd 1), KY020053 (isolate 2, herd 1), KY020054 (isolate 3, herd 1), KY020055 (isolate 4, herd 2) KY020056 (isolate 5, herd 3), KY020057 (isolate 6, herd 4),

KY020058 (isolate 7, herd 5), KY020059 (isolate 8, herd 6), KY020060 (isolate 9, herd 7), KY020061 (isolate 10, herd 8), KY020062 (isolate 11, herd 8), KY020063 (isolate 12, herd 9), KY020064 (isolate 13, herd 10), KY020065 (isolate 14, herd 11), KY020066 (isolate 15, herd 12), KY020067 (isolate 16, herd 13), KY020068 (isolate 17, herd 14), KY020069 (isolate 18, herd 15), KY020070 (isolate 19, herd 16), KY020071 (isolate 20, herd 17), KY020072 (isolate 21, herd 18), KY020073 (isolate 22, herd 19), KY020074 (isolate 23, herd 20), KY020075 (isolate 24, herd 20), KY020076 (isolate 25, herd 21), KY020077 (isolate 26, herd 22).

Results

In this study, phenotypic and genotypic approaches were used. Phenotypic tests were followed in order to isolate and identify *S. aureus* strains in all isolates exploited. Moreover, the PCR methods were performed to check presence of *blaZ* and *mecA* resistance genes. All staphylococcal strains studied were extracted directly from samples milk received, during several years, from cows suffering of clinical mastitis and also from cows with subclinical mastitis coming all from several farms situated in North of Portugal. Identification of these strains was carried using phenotypic tests as cited in **Table 10**. Phenotypic methods of identification have firstly classified the staphylococcal strains as coagulase positive, which have also showed in parallel that all 26 isolates included in this study have still colonized by other micro-organisms with *S. aureus* like strains N°1 from farm N°1, N°5 from farm N°3 and N°20 coming from farm N°17 were also discovered a presence of coagulase negative staphylococci in parallel with *S. aureus* display. However, strain N°9 received from farm N°7 with *S. aureus* two others microorganisms (*Yeasts* and *Citrobacter freundii*) while Yeast bacteria was also found in strain N°19 (farm N°16) and N°26 (farm N°22) in the same time *with S. aureus*. Finally, the microorganisms as *Klebsiella* and *streptococcus dysgalactiae* were also found in parallel with *S. aureus* into strains N°22 coming from farm N°19 and strain N°25 received from farm N°21, respectively (**Table 10**).

Table 10: Mastitic milk samples information and phenotypic tests performed for *S. aureus* isolates collected during 2003-2004 and 2007-2008 periods.

[a]Number of isolates	Source and year of collection of isolates		Phenotypic tests						
	Number of herd	Year	[b]Blood agar	Gram taining	Coagulase	Catalase	VP	[c]Slidex Staph Kit
1****	1	2003	+	G^+C	+	+	+	+
2	1	2008	+	G^+C	+	+	+	+
3	1	2008	+	G^+C	+	+	+	+
4	2	2003	+	G^+C	+	+	+	+
5****	3	2004	+	G^+C	+	+	+	+
6	4	2003	+	G^+C	+	+	+	+
7	5	2003	+	G^+C	+	+	+	+
8	6	2003	+	G^+C	+	+	+	+
9***, *****	7	2004	+	G^+C	+	+	+	+
10	8	2003	+	G^+C	+	+	+	+
11	8	2003	+	G^+C	+	+	+	+
12	9	2003	+	G^+C	+	+	+	+
13	10	2004	+	G^+C	+	+	+	+
14	11	2003	+	G^+C	+	+	+	+
15	12	2003	+	G^+C	+	+	+	+
16	13	2003	+	G^+C	+	+	+	+
17	14	2004	+	G^+C	+	+	+	+
18	15	2003	+	G^+C	+	+	+	+
19***	16	2004	+	G^+C	+	+	+	+
20****	17	2007	+	G^+C	+	+	+	+
21	18	2008	+	G^+C	+	+	+	+
22*,**	19	2008	+	G^+C	+	+	+	+
23	20	2008	+	G^+C	+	+	+	+
24	20	2008	+	G^+C	+	+	+	+
25**	21	2008	+	G^+C	+	+	+	+
26***	22	2008	+	G^+C	+	+	+	+

[a]Other microorganisms identified during the milk samples microbiologic analysis: **Klebsiella spp.*; ***Streptococcus dysgalactiae.*; ****Yeasts*; *****Coagulase-negative staphylococcus*; ******Citrobacter freundii*.

[b]Blood agar - Columbia ANC agar supplemented with 5% of sheep blood (bioMérieux, Marcy l'Etoile, France).
cSlidexTM Rapid Staph Latex Kit (bioMérieux, Marcy l'Etoile, France).
VP, Voges-Proskaeur.
G^+C, Gram positive cocci.
+, Positive test.Table 1 and 2 depicted the phenotypic and genotypic results relative to the detection of the resistance or susceptibility to Beta-lactams family antibiotics.

Phenotypic assessment of beta-lactams resistance

The results obtained of antimicrobial susceptibility test demonstrate that the resistance to aztreonam (Monobactames group) was noted in all of isolates (n=26; 100.0%), whereas the resistance to penicillin (Penicillins G group) was found considerably higher arriving at 76.9% (n=20) followed by resistance to ampecillin (Aminopenicillins group) with 73.0% (n=19). The susceptibility test performed for others antibiotics within this group showed that one only strain was resistant to oxacillin and amoxicillin (n=1; 3.8%) while all rest of strains were sensible 100.0% (n=26) to amoxicillin + gluvalunic acid; in the same time, the same one strain resistant to oxacillin was also found resistant to Amoxicillin (Penicillins M group) presenting 3.8% (n=1). Unlike, all strains showed a significantly higher susceptibility 100.0% (n=26) for others antimicrobial groups studied in parallel as cefazolin (Cephalosporines group); piperacillin (Ureidopenicillins group); imipenem (Carbapenems group) and ticarcillin (Carboxypenicillins group) (**Table 11**).

Table 11: Antimicrobial tests performed to all *S. aureus* isolates from bovine mastitis collected during 2003-2005 and 2007-2008 periods against β-lactam antibiotics by the disk diffusion method following CLSI guidelines.

Number Of isolates	B-lactam antibiotics (µg in disk)									
	AZT (30)	PEN (6)	AMP (10)	OXA (1)	AMX (25)	AMC (20+10)	CFZ (30)	PIP (100)	IPM (10)	TIC (75)
1	R	R	R	S	S	S	S	S	S	S
2	R	S	S	S	S	S	S	S	S	S
3	R	R	R	S	S	S	S	S	S	S
4	R	R	R	S	S	S	S	S	S	S
5	R	R	R	S	S	S	S	S	S	S
6	R	R	R	S	S	S	S	S	S	S
7	R	R	R	S	S	S	S	S	S	S
8	R	R	R	S	S	S	S	S	S	S
9	R	R	R	S	S	S	S	S	S	S
10	R	R	R	S	S	S	S	S	S	S
11	R	R	R	S	S	S	S	S	S	S
12	R	S	S	S	S	S	S	S	S	S
13	R	R	R	S	S	S	S	S	S	S
14	R	R	R	S	S	S	S	S	S	S
15	R	R	R	S	S	S	S	S	S	S
16	R	R	R	S	S	S	S	S	S	S
17	R	R	R	S	S	S	S	S	S	S
18	R	R	R	S	S	S	S	S	S	S
19	R	R	S	S	S	S	S	S	S	S
20	R	R	R	S	S	S	S	S	S	S
21	R	S	S	S	S	S	S	S	S	S
22	R	R	R	S	S	S	S	S	S	S
23	R	S	S	S	S	S	S	S	S	S
24	R	S	S	S	S	S	S	S	S	S
25	R	R	R	R	R	S	S	S	S	S
26	R	S	S	S	S	S	S	S	S	S
%Resistance	**100.0**	**76.9**	**73.0**	**3.8**	**3.8**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

R, Resistant strains; S, Sensible strains.

Presence of the *blaZ* gene in staphylococci of mastitis origin

The PCR method was carried in order to detect presence of *mecA* and beta-lactamases encoded by *blaZ* gene (**Figure 41**). According to PCR results, all *S. aureus* strains retrieved from mastitis milk samples carrying *blaZ* gene that was performed through successful amplification of (377bp) specific products (**Table 12**, **Figure 42**), and all these strains are negative for *mecA* genes, except for isolate 25 that was the only one positive for *mecA* (**Table 12**, **Figure 43**).

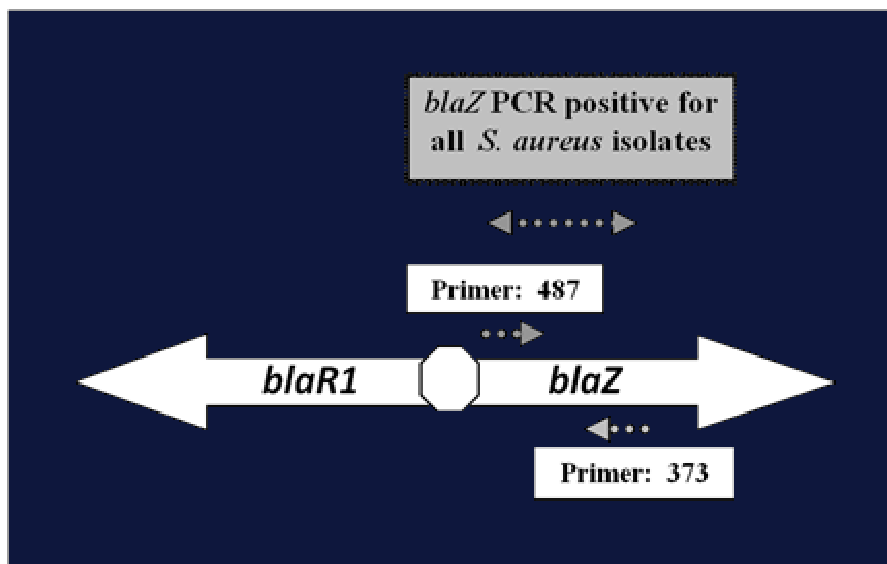

Figure 41: Structure of the sequenced fragment encoding the *blaR1* and *blaZ* region.

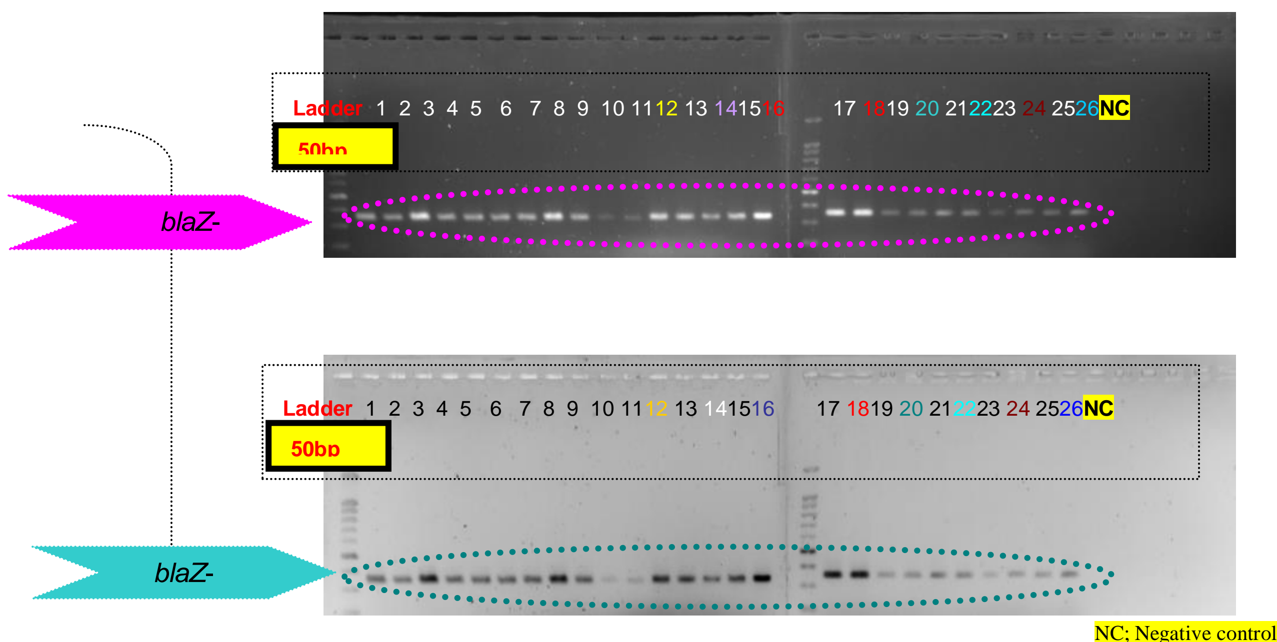

Figure 42: Results retained on agarose gel after amplification of *blaZ* gene by using PCR method.

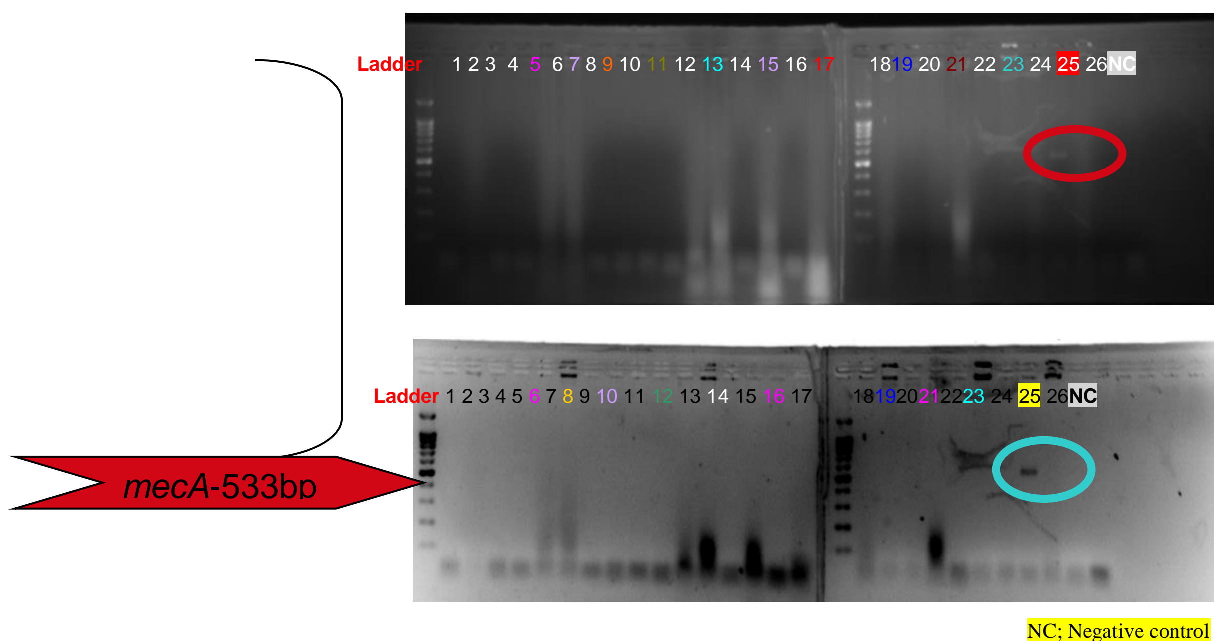

Figure 43: Results retained on agarose gel after amplification of *mecA* gene by using PCR method.

Table 12: Antibiotic resistance genes profile (*blaZ* and *mecA*) of the tested *S. aureus* isolates from bovine mastitis by PCR during 2003-2004 and 2007-2008 periods.

	Isolates tested																										
Genes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	%
blaZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**100.0**
mecA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	**3.8**

Comparison of proportion of resistance genes and of phenotypic resistance

The findings confirmed that the proportion of strains mastitis bovine with phenotypic resistance in this current study did not concurred with the proportion of those identified with the chosen resistance genes (**Table 13**). The selected resistance genes (**Table 12**) are identified in all strains of *S. aureus*, but all of these strains were not phenotypically resistant to Beta-lactams (**Table 11**).

Table 13: Relationship between the phenotypic and genotypic antibiotic profile of the tested *S. aureus* isolates during 2003-2005 and 2007-2008 periods.

Number Of isolates	B-lactam antibiotics (µg in disk)										Resistance Genes	
	AZT (30)	PEN (6)	AMP (10)	OXA (1)	AMX (25)	AMC (20+10)	CFZ (30)	PIP (100)	IPM (10)	TIC (75)	*BlaZ*	*mecA*
1	R	R	R	S	S	S	S	S	S	S	+	-
2	R	R	R	S	S	S	S	S	S	S	+	-
3	R	R	R	S	S	S	S	S	S	S	+	-
4	R	R	R	S	S	S	S	S	S	S	+	-
5	R	R	R	S	S	S	S	S	S	S	+	-
6	R	R	R	S	S	S	S	S	S	S	+	-
7	R	R	R	S	S	S	S	S	S	S	+	-
8	R	R	R	S	S	S	S	S	S	S	+	-
9	R	R	R	S	S	S	S	S	S	S	+	-
10	R	S	S	S	S	S	S	S	S	S	+	-
11	R	R	R	S	S	S	S	S	S	S	+	-
12	R	R	R	S	S	S	S	S	S	S	+	-
13	R	R	R	S	S	S	S	S	S	S	+	-
14	R	R	R	S	S	S	S	S	S	S	+	-
15	R	R	R	S	S	S	S	S	S	S	+	-
16	R	R	R	S	S	S	S	S	S	S	+	-
17	R	R	R	S	S	S	S	S	S	S	+	-
18	R	R	S	S	S	S	S	S	S	S	+	-
19	R	S	S	S	S	S	S	S	S	S	+	-
20	R	R	R	S	S	S	S	S	S	S	+	-
21	R	S	S	S	S	S	S	S	S	S	+	-
22	R	S	S	S	S	S	S	S	S	S	+	-
23	R	S	S	S	S	S	S	S	S	S	+	-
24	R	R	R	S	S	S	S	S	S	S	+	-
25	R	R	R	R	S	S	S	S	S	S	+	+
26	R	S	S	S	S	S	S	S	S	S	+	-
%Resistance	100.0	76.9	73.0	3.8	3.8	0.0	0.0	0.0	0.0	0.0	100.0	3.8

Sequencing analysis of *blaZ*

The amplicons of all the positive isolates were sequenced. The retrieved sequences were analyzed and a consensus sequence for each isolate was created. When blastn of nucleotide consensus sequences were conducted in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), similarities of 100.0–99.0% and e-values of 0.0 were shared for *S. aureus* strains (**Table 14**). This data supported the selection of the *S. aureus* strains used in the phylogenetic analysis.

Table 14: Blastn between consensus sequences of all tested bovine mastitis *S. aureus* isolates in the NCBI-GenBank database. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Isolate	GenBank Acession no.	Strain	Position	E-value	% Identities
1	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008879	0.0	100.0
2	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
3	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008865	0.0	100.0
4	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
5	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008879	0.0	99.0
6	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
7	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
8	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
9	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
10	DQ016066	*Staphylococcus aureus* strain A1358_1	181 – 544	0.0	100.0
11	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
12	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
13	DQ016066	*Staphylococcus aureus* strain A1358_1	181 – 543	0.0	100.0
14	CP011528	*Staphylococcus aureus* strain RKI4	1929299 – 1929676	0.0	99.0
15	CP012971	*Staphylococcus aureus* strain ST20130939	13433 – 13797	0.0	99.0
16	CP012971	*Staphylococcus aureus* strain ST20130939	13433 – 13797	0.0	99.0
17	CP012971	*Staphylococcus aureus* strain ST20130939	13420 – 13797	0.0	99.0
18	CP012971	*Staphylococcus aureus* strain ST20130939	13420 – 13797	0.0	99.0
19	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
20	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
21	CP011528	*Staphylococcus aureus* strain RKI4	1929299 – 1929676	0.0	99.0
22	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
23	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
24	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
25	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
26	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0

Phylogenetic analysis

The consensus nucleotide sequences of *blaZ* were aligned using Clustal W through MEGA version 5.0 (**Kimmura, 1980**) for phylogenetic inference. An evolutionary history was inferred using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model (**Tamura *et al.*, 2011**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1.000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using the MCL approach. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total

of 293 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0 (**Kimmura, 1980**).

For the phylogenetic analysis, the retrieved sequences were aligned with human, bovine and animal food *S. aureus* sequences, animal *S. warneri* and *S. intermedius* sequences, and with one *S. haemolyticus* sequence that was used as an outgroup. For all selected strains respective Genbank accession nos. are referred in the phylogenetic tree.

Phylogenetic analysis of *blaZ*

Phylogenetic relationships were inferred using the ML method as described in methods section. The phylogenetic analysis placed the 26 *S. aureus* isolates in 3 different clusters, supported by strong bootstrap values of 94.0%-95.0% (of 1.000 replicates) (**Figure 44**). The isolates 1, 3-10, 11, 13, 19, 20 and 22-26 were placed in cluster 1, supported by bootstrap values of 62.0% (of 1.000 replicates), and are more closely related to bovine and human *S. aureus* strains GenBank accession nos. AY369345, AY369348, CP010940, DQ016066, CP013619 and DQ016047 (**Figure 44**). Moreover, a subcluster inside cluster 1 was formed, supported by strong bootstrap value of 86.0% (of 1.000 replicates), were isolates 14 and 21 (similarly related and supported by bootstrap value of 76.0% (of 1.000 replicates)) are closely related to human *S. aureus* RKI4 strain (GenBank accession no. CP011528) (**Figure 44**). In cluster 2, isolate 2 appears as single and is the most divergent strain of all tested isolates. Isolate numbers 15-18 were placed in the 3rd cluster, supported by bootstrap values of 61.0% (of 1.000 replicates), and are more closely related to bovine and human GenBank accession nos. AP004832, DQ016055, CP002115 and CP012971 (**Figure 44**).

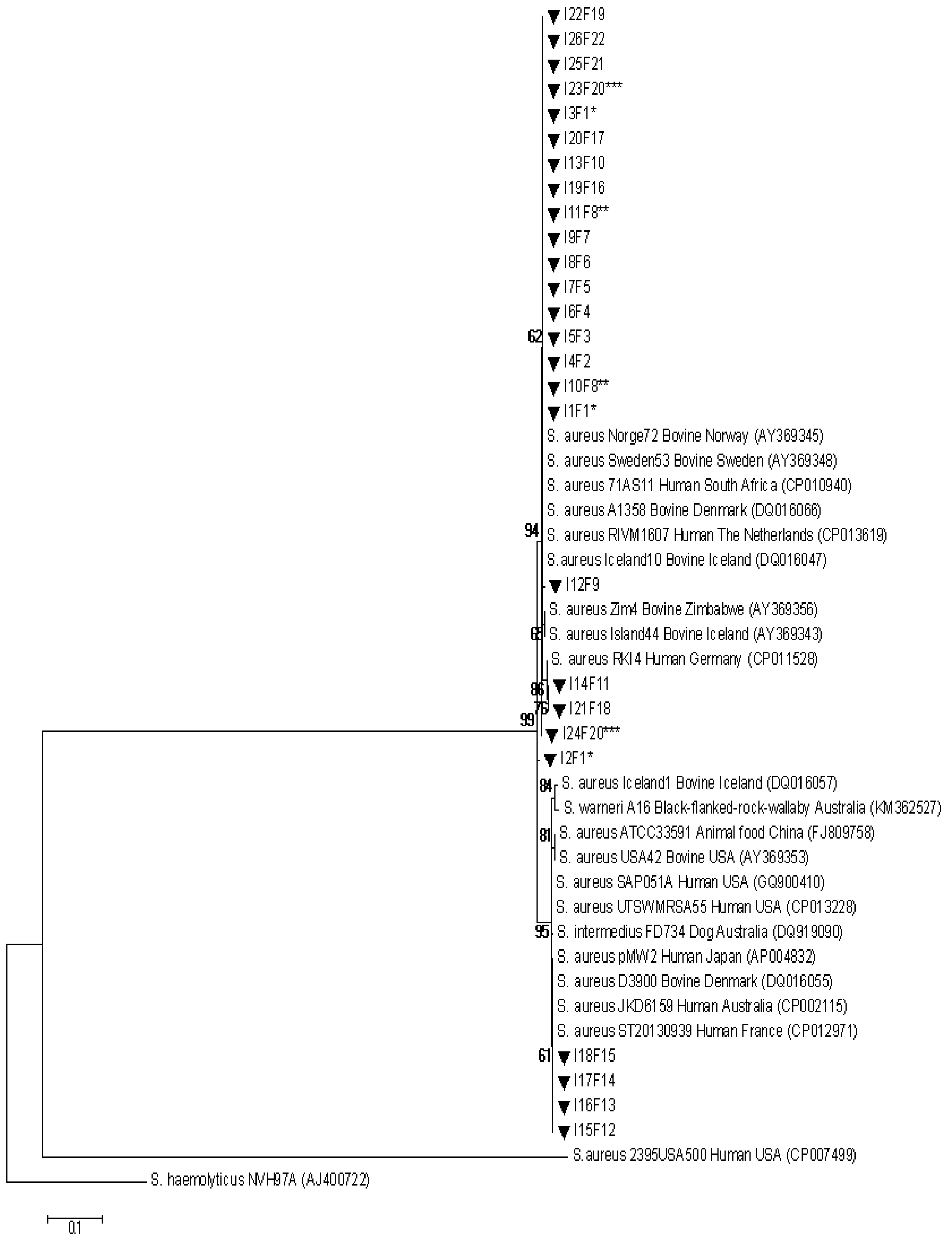

Figure 44: Phylogenetic analysis of sequences of *blaZ* region in bovine mastitis *S. aureus* isolates. ML method was inferred. Bootstrap resampling was used to determine the robustness of branches; values from 1,000 replicates are shown. Filled triangle, indicates the 26 bovine mastitis *S. aureus* isolates tested in this study.

Discussion

Bovine mastitis is known as the most common, economically unbearable and complicated disease facing the dairy farmer. Of interest, *S. aureus* is considered as the most frequent causative organism of bovine mastitis (**Berghash *et al.,* 1983; Wyder *et al.,* 2011**) and whereas Beta-lactam antimicrobials are widely recommended in therapy of cattle, particularly for the treatment of mastitis (**Gianneechini *et al.,* 2002; Fejzi *et al.,* 2014**) and are the most frequently used drugs in veterinary medicine (**Pitkala *et al.,* 2007**). In this regards, penicillin, amoxicillin/clavulanic acid, and ampicillin are the most commonly used agents for treatment of this disease caused by *S. aureus*, due to their low resistance rates and narrow spectra **(Gianneechini *et al.,* 2002)**.

The present study is on line of this problem concerning antimicrobial resistance in the case of bovine mastitis. It reports on the situation in the North of Portugal. Even if the studies of the resistances of *S. aureus* causing bovine mastitis in Portugal appear rare, trends in antibacterial resistance of major bovine mastitis pathogens in Portugal have increased in dairy farms in the northwestern, central and southern regions of Portugal (**Rocha *et al.,* 2014**). Interestingly, the presented results indicated a considerable prevalence of a set group Beta-lactams antibiotics resistant strains among *S. aureus* isolated from bovine mastitis in the North of Portugal. A second work that have been done on 30 *S. aureus* isolates from subclinical mastitis in 7 farms from the central region of Portugal show that the resistance to penicillin stay less than our reported finding. In this study, **Nunes *et al.*, (2007)** showed that the production of Beta-lactamase was detected in 20 isolates (66.7%), all of which were resistant to penicillin. They noted also that the fact that none of the susceptible isolates to penicillin of *S. aureus* were βeta-lactamase producers indicate that the high levels of penicillin-resistance might be attributed to the production of these enzymes. Compared to other reports in the world, our findings showed similarities or differences with other studies depending on the world region. Thus, the highest levels were observed in case of resistance to Monabactams group (case of aztreonam 100.0%) and for both groups of Penicillin G (case of penicillin (76.9%)) and Aminopenicillins (case of ampIcillin (73.0%)) of family's Beta-lactams; these levels were often higher in the comparison with the general trend observed worldwide. However, Penicillins M group (case of oxacillin and of amoxicilin) was almost susceptible against *S. aureus* (96.2%) whereas all rest of antibiotics group that were tested from same family of Beta-lactams were presented a higher rate (100.0%) of susceptibility to destruction of *S. aureus* causing mastitis bovine, as case of Ureidopenicillins group (piperacillin case), Carboxypenicillins group (ticarcillin case),

Carbapenems group (imipenem case) and group of Cephalosporines (cefazolin case). Interestingly, the results of this research reveal also that all 26 staphylococcal isolates extracted from mastitis bovine were carrying *blaZ* gene detected by PCR method and all these strains are in the same time negative for *mecA* gene, except for isolate 25 that was the only one positive for *mecA* gene.

Twenty of 26 isolates showed an increase in resistance to penicillin 76.9%; and the same strains, except N°18 from farm 15, were also resistant to ampicillin 73.0% (19/26). One isolate, N°25 from farm 21 was found both resistant to oxacillin and amoxicillin with 3.8% in our study. Conversely, the percentage of resistance that was observed in the case of Beta-lactam antibiotics in bovine mastitis in Eastern Poland was lower than our results obtained, the resistance observed to penicillin and ampicillin was 23.6 and 22.8%, respectively; whereas amoxicillin resistance was lower in case of our strains (3.8%) in comparison with Poland strains when amoxicillin is considered (17.9%) (**Szweda *et al.,* 2014**). In October 2014, year of both studies; **Jagielski *et al.*, (2014)** found high level than the first noted in Eastern Poland, but stays also less than our rates observed of the penicillin resistance that have been increased to reach 41.0% among of 80 *S. aureus* mastitis isolates collected from 14 dairy farms in Poland. This drug along with other β-lactamase-sensitive Beta-lactams should rather not be considered for the treatment of bovine mastitis caused by *S. aureus*, **Jagielski *et al.*, (2014)** added.

On the other hand, **Klimienė *et al.*, (2011)** indicated in both studies performed in 2011, that among 506 strains isolated from sub-clinical and clinical cases of bovine mastitis in Lithuania were showed rates a bit high than our findings of resistance to penicillin (81.6%), ampicillin (83.2 %) and much different for amoxicillin (81.7%). The second work of **Klimienė *et al.*, (2011)** shows approximately the same rate of resistance with a little bit of reduction in level of amoxicillin (81.3%), penicillin (76.7%) and ampicillin (78.4%). Thus, these findings showed a similar level for our study for penicillin and almost level for ampecillin with a great difference for amoxicillin that appears inactive more against *S. aureus* causing mastitis bovine in farms herds in Lithuania than level found in Portugal farms. This highest rate resistance observed could be related to a great capacity of spread of *S. aureus* resistant strains within bovine farms in both countries.

Different proportion marked of resistance against Beta-lactam antibiotics were also encountered in numerous different geographical regions principally in the Europeans countries. For example, in Estonia, the penicillin resistance that was reported by **Kalmus *et al.*, (2011)** is around 61.4% within 8204 samples investigated from clinical and sub-clinical mastitis in

Estonian dairy farms. Nevertheless, in Finland, 52.1% of *S. aureus* strains isolated from clinical and sub-clinical mastitis samples were resistant to penicillin (**Pitkala *et al.,* 2004**).

In the last year, **Petrovski *et al*., (2015)** compared the antimicrobial susceptibility patterns of three common mastitis pathogens (*Staphylococcus aureus*, *Streptococcus uberis* and *Str. dysgalactiae*) isolated from milk samples from New Zealand and the USA. The results retrieved from this study showed that the non-susceptible isolates of *S. aureus* were identified for amoxicillin, ampicillin and penicillin: 20.6% and 36.0% for New Zealand and the USA, respectively. Therefore, these findings are much lower than our results. So, this observed difference might be also related to use of drugs in each country separately because the choice of antimicrobial agent depends on availability and regulations, which differ between countries. Moreover, in France, the overall proportion of antibiotic resistance was low, except for penicillin G in staphylococci (**Botrel *et al.,* 2010**). However, in the Turkey, the highest resistance was observed in 63.3% of the strains against Beta-lactam antibiotics, penicillin and ampicillin while no resistance was detected for amoxicillin-clavulanate, oxacillin. Resistance against Beta-lactams in this country has increased from 43.5% in 1995 to 58 to 77.0% from 1999 to 2004 (**Güler *et al.,* 2005**). In 2015, **Thomas *et al*., (2015)** found a rate 36.0% of *S. aureus* that were resistant to penicillin G in mastitis pathogens isolated from acute cases of clinical mastitis in dairy cows across Europe.

When Africa is considered, the resistance of isolates of *S. aureus* isolated from mastitis bovine in farms dairy in South West Ethiopia was detected a high percentage than our results obtained for penicillin 76.9% instead 87.2%, 3.8% instead 46.0% for amoxicillin (**Sori *et al.,* 2011**).

Parallelly, compared to findings in America Latin's countries, our findings show noticeable differences with reports in Argentina dairy herds, as the antimicrobial susceptibility of *S. aureus* causing bovine mastitis to penicillin was 48.4% and no resistance observed to oxacillin while the resistance β-lactamase activity was detected in 89.0% of 46 penicillin-resistant strains. Apart from penicillin, antimicrobial resistance in *S. aureus* causing bovine mastitis remains rare in Argentine dairy farms (**Russi *et al.,* 2008**). Similar rate was reported also in Argentina in 2002 by **Calvinho *et al*., (2002)** showing that the highest levels of resistance were against penicillin and ampicillin (47.6%) whereas no resistant strains against oxacillin were detected. Apparently, we can notice that the levels reported in these both studies published in 2008 and 2002 could be classified as higher than those reported **Gentilini *et al*., (2000)** in 2000 where only 40.3% for this type of resistance in case of *S. aureus* isolated from bovine clinical and sub-clinical mastitis is observed in Argentina during 1996 to 1998.

Taken in their overall, our results remained also more high than those obtained in staphylococci isolated from sub-clinical and clinical cases of bovine mastitis from the west littoral region of Uruguay, in which the resistance to penicillin was observed similar to the results obtained in case of isolates from Argentina in 2002 **(Calvinho *et al.*, 2002)** and 2008 (**Russi *et al.,* 2008**), such as 47.6% and 46.7% cases into 160 and 157 of 336 strains of *S. aureus* were resistant to penicillin and ampicillin, respectively; in case of bovine mastitis from Uruguay (**Gianneechini *et al.,* 2002**). In the Northeast of Brazil; **Da Costa *et al*., (2015)** showed that in the test for susceptibility to antimicrobials encountered in 2064 milk samples of 525 lactating cows whose 57.8 % were only characterized as *S. aureus*, 28.0% as coagulase-positive staphylococci other than *S. aureus* (oCPS), and 14.2% as coagulase-negative staphylococci (CNS) showed amoxicillin (32.6%) to be the less effective drug in vitro in opposition to our findings in North Portugal. The genotypic characterization showed that 93.1% of the samples were tested positive for the *blaZ* gene, while we have identified the presence of *blaZ* in all strains in our case. **França *et al*., (2012)** determined in their study entitled - Antimicrobial resistance of *Staphylococcus* spp. from small ruminant mastitis in Brazil- that almost isolates were most resistant to amoxicillin (50.0%). Although phenotypic resistance to oxacillin was observed in 12.8% of the isolates, none harbored the *mecA* gene that is responsible of resistance to oxacillin. While, the staphylococci resistance to Beta-lactams is related with production of Beta-lactamase in 45.7% isolates harbored *blaZ* gene that appears the main mechanism responsible of resistance to Beta-lactams and they show that this resistance may be associated with the use of antimicrobial drugs without veterinary control. These different findings especially for amoxicillin and oxacillin resistance could also be explained by difference in capacity to resistance among each Staphylococcal species causing mastitis disease.

Finally, in Asia, **Alekish *et al*., (2013)** showed in Jordan in 2013 a high prevalence of resistance which reached 84.5% for penicillin within 205 bovine mastitis pathogens. In South India, the resistance rate of *S. aureus* from sub-clinical bovine mastitis to penicillin and ampicillin was 41.4% and 3.9%, respectively. These results are lower than our findings obtained for these drugs, especially for ampicillin (**Muhamed *et al.,* 2012**) whereas **Jagadeeswari *et al*., (2013)** showed a high level in other study in India (Coimbatore province), in that 80.0% of isolates of *S. aureus* extracted from bovine mastitis were resistant to penicillin with high rate of Beta-lactamase activity and resistant to other Beta-lactam antibiotics like ampicillin, amoxicillin. The highest rate of resistance of *S. aureus* causing bovine mastitis to penicillin (96.3%) was registered in a single herd in China as described by **Gao *et al*., (2012)**.

Taken in their totality, all these studies cited above indicate clearly the presence of antimicrobial concern with regard to bovine mastitis. Furthermore, these antimicrobials resistance is linked exclusively to *Blaz* gene. This is of high interest as resistance of bacteria against Beta-lactam antimicrobials is known to show serious increasing at a significant level that has become a common problem in primary care medicine. There are numerous mechanisms of antimicrobial resistance to Beta-lactam antibiotics (**Mandell *et al.,* 1996; Mcmanus *et al.,* 1997; Keith *et al.,* 2000**). One most important mechanism is that associated with the production of Beta-lactamases (**Bush *et al.,* 1995; Keith *et al.,* 2000**); while others factors that could involved in this resistance against a particular antibiotic in a specific region may be due to its frequent and long-term utilization (**Sabour *et al.,* 2004; Moon *et al.,* 2007; Kumar *et al.,* 2010; Sharma *et al.,* 2015**).

It appears from all the studies published in this topic, that the prevalence of resistance to antibiotics (case of penicillin and ampecillin) is higher in case of bovine mastitis caused by *S. aureus*. Authors in most cases explained these high rates encountered in mastitis bovine causing with production of Beta-lactamase encoded by the gene *blaZ* (**Szweda *et al.,* 2014**). As, **Watts *et al*., (1997)** determined that *S. aureus* isolated from bovine intramammary infections that of the five beta-lactam compounds tested, penicillin and ampicillin were most affected by Beta-lactamase activity.

In general, this penicillin resistance showed in *S. aureus* is conferred by two mechanisms well known. The first mechanism is considered as a most important mechanism of resistance to penicillin and is associated directly with expression of a Beta-lactamase, which can hydrolyse the antibiotic so rendering it inactive (**Hartman *et al.,* 1984**) (example of inactivation of penicillin by hydrolysis of its Beta-lactam ring). The second one is primarily related with human isolates and is responsible to resistance due to a penicillin-binding protein 2a, PBP2a, encoded by *mecA* gene that plays a role in methicillin resistance, which is a much less sensitive target than the wild-type PBPs (**Hartman & Tomasz, 1984; Deurenberg *et al.,* 2007; Hata, 2016**). A great variety of strategies of resistance to eubacteria challenged by Beta-lactams have been also discovered such as the acquisition of an additional low-affinity PBP, the overexpression of an endogenous low-affinity PBP, the alteration of endogenous PBPs by point mutations or homologous recombination or a combination of the above (**Zapun *et al.,* 2008**). Among the genes variants encoding Beta-lactamases. There are four classes of *blaZ* product (A, B, C, D) (**Kernodle *et al.,* 1989; Dyke, 1997**). For example, the genes variants encoding the classes A, C and D Beta-lactamase and the classe B Beta-lactamase are located on plasmid and chromosome, respectively (**Livermore, 1995**). This class acts by a serine-based mechanism

and hydrolyse its substrates through a serine active site, while type B or metallo-β-lactamasesare metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis (**Ambler, 1980; Bush & Jacoby, 2010**). In relation to the influence of the localization of the *blaZ* gene on antimicrobials resistance. **Bagcigil *et al*., (2012)** demonstrated that the plasmid location of the *blaZ* gene was not statistically significantly more common in samples from Finland than those obtained Sweden, and hence does not explain the higher proportion of penicillin-resistant isolates of *S. aureus* causing bovine mastitis in Finland compared to Sweden.

Stress also plays vital in achieving the cows by mamite, more an animal suffers stress in its environment, the less efficient its immune system is, and the less it can resist microbial invasions. So, the more stress, the more chances of mastitis increases. **Giesecke *et al*., (1985)** even shown that stress affects the integrity of intramammary cells, which is one more factor which promotes mastitis. The following are some sources of stress: Excessive density of animals; proximity of cows encourages microbial exchanges and tense relations between animals; irregular management, unpredictable behaviour on the part of the farmer; Noise and Stray voltage.

Taken together, our results in combination with the others, it could be suggested that the use of an in vitro beta-lactamase test for determining resistance to penicillin G and for ampicillin of staphylococci before any starting of treatment of mastitis bovine will be recommended with regard to the resistance level encountered of these both antimicrobial agents in order to avoid this phenomenon of resistance.

Comparison of occurrence of resistance genes and phenotypic resistance

In veterinary medicine, penicillin is referred as the first choice for treatment of bacteria that are inherently sensitive to it. Therefore, penicillin resistance identification tests are important in veterinary medicine (**Pitkälä *et al.,* 2007**). The identification of the *blaZ* gene which encodes the production of β-lactamase by PCR methods was taken as a reference method (**Pitkälä *et al.,* 2007; Lowy, 2003; Haveri *et al.,* 2005**).

In our case 100.0% strains was positive for beta-lactamase but the susceptibility testing was detected just in 76.9% and 73.0% of the strains that were susceptible for penicillin and ampicillin, respectively. Therefore, these findings confirmed that the proportion of strains mastitis bovine with phenotypic resistance in this current study did not concurred with the proportion of those identified with the chosen resistance genes **(Table 13)**. This phenomenon

could be associated to numerous alternatives as to why findings of genotypic testing may differ from findings of phenotypic susceptibility testing. According to **CLSI (2014)**, if Beta-lactamase positive, report as resistant to penicillin and ampicillin, it is not excluded that β-lactamase–negative isolates may be resistant to penicillin and ampicillin by other mechanisms.

To our knowledge, there are not enough data explaining this phenomenon whereas there are some information trying only to explain how the non correspondence exists between isolates with phenotypic resistance and the proportion of detected resistance genes. In our case this non correspondence observed could be explained by several mechanisms, for example the possibility of the presence of a mutation or a set of mutations at the level of the gene encoded for this resistance. For why, the gene is identified but it became no expressed towards the penicillin and ampicillin resistance, which may be due to these mutations.

A recent data reported by **Tasara *et al.*, (2013)** showed out of 78 *S. aureus* strains retrieved from bovine mastitis milk and 10 of them carried *blaZ*, *blaI* and *blaR* genes. Among of these 10 strains, 5 strains were phenotypically resistant to penicillin while the other 5 (all belonging the clonal complex 8) were susceptible to penicillin. The presence of the *blaZ*, *blaR* and *blaI* genes in all 5 strains were confirmed by PCR method while the sequencing results of these genes uncovered a 29 base deletion within the *blaZ* gene in all these strains that cause a translational frame shift, which is predicted to induce abrogation of *blaZ* expression. Further, single nucleotide insertions and deletions were discovered also in *blaR* of 3 strains. Utilization of the genetically altered *blaZ* genes detected as targets, a real time PCR system for detecting CC8 associated *blaZ* positive *S. aureus* strains that still remain susceptible to penicillin was developed. Such strains are part of detection challenges that must be considered in routine application of genotypic resistance testing of bovine mastitis *S. aureus* **(Tasara *et al.*, 2013)**.

Ruegg *et al.*, (2015) showed that the proportion of isolates with phenotypic resistance, in opposition to our findings did not correspond to the proportion of detected resistance genes. They are associated this phenomenon with multiple mechanisms. For example, the researchers use usually only a test for a limited number of genes (case of macrolide-lincosamide-streptogramin resistance which is associated to 21 genes). Another possibility could be related with mutation of the primer annealing site, as hypothesized by **Haveri *et al.*, (2005)**. In addition, they suggested still that differences in detection of the *blaZ* gene and the phenotypic development of resistance may be due to utilization of incorrect resistance breakpoints. Therefore, the question of accuracy and reliability of the followed methods and tests must be taken into consideration.

It appears from our findings that the identification of the gene does not mean automatically that the antimicrobial resistance exists. For this reason, we agree with **Haveri *et al*., (2005)** that suggested that phenotypically susceptible isolates that carry resistance genes should be considered as potentially resistant.

In relation to group of Aminopenicillins, our findings showed a higher rate of resistance just to ampecillin as already cited while the resistance to amoxicillin was also noted in one only strains. No strain was found resistant to amoxicillin/clavulanic acid that **Malinowski *et al*., (2008)** showed that it was among the most active antibiotics tested against *S. aureus* with 97.4%. The same strain that encountered resistant to penicillin, ampicillin and for amoxicillin was also resistant to oxacillin (group of Penicillins M). The resistance of *S. aureus* to oxacillin is due to the acquisition of the *mecA* gene (**Al-akydy *et al.,* 2014**).

The resistant to aztreonam antibiotic was noted in all these strains 100.0%. The resistance appeared to aztreonam in all strains showed that it is not might be related with presence of *blaz* gene encoded Beta-lactamase; but because the aztreonam is relatively inactive against gram-positive and anaerobic bacteria whereas it is extremely effective against aerobic gram-negative bacteria, even in low concentrations (**Sykes & Bonner, 1985; Harold & Neu, 1990**). In addition, it is highly resistant to enzymatic hydrolysis by Beta-lactamases and demonstrates a high degree of stability against plasmid-mediated gram-negative lactamases. Its pharmacologic profile can be attributed to its unique chemical properties and mechanisms of action.

The remaining groups of antibiotics tested in the family of Beta-lactams namely piperacillin (Ureidopenicillins group), imipenem (Carbapenems group), ticarcillin (Carboxypenicillins group) and cefazolin (Cephalosporin group). All these antibiotics showed high activity against *S. aureus* isolated from bovine mastitis. To our knowledge, there are not an enough studies that focused on these antimicrobials. In 2015, **Sharma *et al*., (2015)** indicated also in vitro antimicrobial sensitivity of isolates from clinical and sub-clinical mastitis. Our findings concerning imipnen (100 percent susceptible to *S. aureus* isolates) are in the same line of these investigations, while they found still a high sensitivity (88.8%) towards piperacillin/tazobactam as they observed a resistance to cefazolin arriving at 33.3% against that we found for cefazolin antibiotic that it was 100.0% susceptible in our case.

In general, the microorganisms resistance of antibiotics has become a serious danger threatening the animal's health as well as human beings health. In Turkey, **Tel *et al*., (2012)** showed in a study done on human and bovine strains that the strains of *S. aureus* retrieved from human were resistant to imipenem 57.8% while no strain of *S. aureus* was found resistant to this antibiotic in case of strains from bovine mastitis. Moreover, the resistance appears to

amoxicillin/clavulanic acid, amoxicillin case of human was 56.1% and 64.9%, respectively; but in case of bovine strains, the resistance to both antibiotics was 1.5% and 0.0%, respectively. The resistance observed to amoxicillin/clavulanic acid and amoxicillin in case of human strains was 56.1% and 64.9%, respectively; but in case of bovine strains, the resistance to both antibiotics was just 1.5% to amoxicillin/clavulanic acid and null 0.0% to oxacillin. In relation to ampecillin and penicillin G resistance in human strains, the resistance was 100.0%, 94.7%, respectively; whereas the resistance to both antibiotics was 100.0% in case of bovine mastitis. Our finding concerning susceptibility with regard to imipenem that showed a great activity to *S. aureus* in case of strains from cattle was similar as that it reported recently by both studies previously described. As, it appears from what indicated by **Tel *et al*., (2012)** that the strains resistance of *S. aureus* isolated from humans was higher than that from cattle, while the resistance to penicillin and ampicillin of *S. aureus* strains of human and cattle origin were absolutely high and is also widespread in comparison with all studies described above. As well as for the amoxicillin/clavulanic acid and amoxicillin resistance was very prevalent among *S. aureus* strains from human origin while it was little or absent between those of cattle origin.

The carboxypenicillins and ureidopenicillins are groups of antibiotics. They belong to the penicillin family and comprise the antibiotics ticarcillin and piperacillin, respectively (**Wright *et al.,* 1999**). These both group of antimicrobial were appeared better activity against *P. aeruginosa*, and are still effective against Gram-negative and Gram-positive bacteria, including enterococci and anaerobic organisms (**Nathwani & Wood, 1993**).

Ticarcillin is used in order to treat moderate-to-severe infections caused by susceptible gram positive and gram negative agents (**Clinical and Research Information, 2016**). Therefore, the activity of ticarcillin that we have observed against to *S. aureus* isolated from mastitis bovine might be related to ticarcillin's antibiotic properties manifests from its capacity to prevent cross-linking of peptidoglycan during the synthesis of cell wall, when the bacteria attempt to divide, causing cell death. Piperacillin has also an action against of the Gram-negative and Gram-positive aerobic and anaerobic bacteria. As other penicillin drugs, piperacillin is activated by binding to specific penicillin-binding proteins (PBPs) localized inside the bacterial cell wall, thereby inhibiting the final step of synthesis of cell wall and leading to autolysis of the bacteria by autolysins.

The *blaZ* gene phylogenetic analysis placed the isolates used in this study in 3 different separated clusters that are closely related to different bovine mastitis and human *S. aureus* strains. It is important to refer that relatively to the isolates 1(2003), 2 (2008) and 3 (2008), belonging to herd 1, that were recovered from mastitic milks, were collected in different

years. From these, isolate 2 (the most divergent in this study) diverged from the other 2 isolates, meaning that the strain in circulation could have genetically changed or that a new strain was introduced into the herd in 2008. Moreover, isolates 10 and 11, belonging to the same year (2003) of milk samples collection, demonstrated genetic similarity between them. Furthermore, isolates 23 and 24 within herd 20, both recovered from milk samples collected in 2008, showed divergence between them, suggesting that different *S. aureus* strains were introduced in the herd or were in circulation at that time. Finally, it is also relevant to refer that within clusters 1 and 3, most isolates are related to a high prevalence of penicillin and ampicillin resistance, and that isolates with a phenotypic susceptibility of 100.0% are included in cluster/subcluster 1 and cluster 2. We were able to detect and sequence *blaZ* gene according to the methods previously described (**Olsen *et al.,* 2006**), however, the evaluated diversity in this study, as described above, was not done in the context of chromosome or plasmid gene location. Studies concerning *blaZ* gene in *S. aureus* isolated from bovine mastitis describing genotyping, diversity, and evolution relatively to gene chromosomally or plasmids location and/or gene encoding penicillin resistance by different methods, such as PCR, PCR-RFLP, PFGE and MLST were previously described (**Olsen *et al.,* 2006; Haveri *et al.,* 2008; Delgado *et al.,* 2011; Bagcigil *et al.,* 2012**).

In summary, this current study indicates that *blaZ* plays a role in Beta-lactam resistance in staphylococci isolated from mastitis bovine inside of farms in North of Portugal; especially in case of penicillin and ampicillin, as it was corroborated by other authors in the field studies. The findings obtained confirmed that the proportion of strains mastitis bovine with phenotypic resistance in this study did not concurred with the proportion of those identified with the chosen resistance genes. In relation to the phenotypic findings obtained from the others groups tested of the family of Beta-lactams namely Ureidopenicillins group (piperacillin), Carbapenems group (imipenem), Carboxypenicillins group (ticarcillin) and Cephalosporin group (cefazolin) have all showed high activity against *S. aureus* isolated from bovine mastitis. The findings from this study indicated that the *S. aureus* strains isolated from mastitis bovine and that it also showed 100 percent the *blaZ* gene are not also resistant 100 percent to penicillin antibiotics and for ampicillin. Finally, we conclude that the *S. aureus* strains are sensitive to others group from Beta-lactam family as they are not always resistant to penicillin antimicrobial and for ampicillin as described by many studies despite the presence of *blaZ* gene. Therefore, it will be necessary to show also the activity of these tested groups apart from ampicillin and penicillin in vivo instead of in vitro because the activity observed in vitro does not ensure efficacy in vivo when treating bovine mastitis. The reason why the antimicrobial susceptibility discovered in vitro has

been considered as a prerequisite for treatment. The utilization of veterinary drugs by veterinarians plays an imperative function and a major role in the prevention and control of diseases in cattle but the practices inside of farms can aid prevent disease and reduce also the need to resort to drugs utilization. However, drugs use is sometimes essential; thus, must be used wisely. Finally, *blaZ* phylogenetic analysis from *S. aureus* isolates shown diversity inside or between different herds in the northwest of Portugal. The evaluation of new bovine mastitis milk samples collected in the same herds, using the same or other methods would be of importance to further discuss the dynamics on resistance patterns of *S. aureus* in the region. So, in order to follow the evolution of resistance to antibiotic groups within Beta-lactam family, such as Penicillin G (penicillin), Penicillin M (oxacillin), Aminopenicillins (ampicillin, amoxicillin associate to clavulanic acid), Ureidopenicillin (piperacillin) and Cephalosporin (cefazolin) in Northern West of Portugal. For this, we have made a new collection of new strains from cows with mastitis caused by *S. aureus* during the year 2017. The same protocol previously described above, for 26 strains that has collected since year 2003 to 2008 from 22 farms, has been saved and followed for the new 26 strains collected in 2017 from 15 farms in the Northern West of Portugal. Thus, the obtained antimicrobial susceptibility and PCR results from this new collected isolates are given in the following tables.

Table 15: Antimicrobial tests performed to all *S. aureus* isolates from bovine mastitis collected during 2017 period against β-lactam antibiotics by the disk diffusion method following CLSI guidelines.

Number Of isolates	B-lactam antibiotics (μg in disk)					
	PEN (6)	AMP (10)	OXA (1)	AMC (20+10)	CFZ (30)	PIP (100)
27	S	S	R	I	I	S
28	S	S	I	I	S	S
29	R	R	R	R	R	S
30	S	S	S	S	S	S
31	S	S	S	S	S	S
32	S	S	S	S	S	S
33	S	S	R	I	S	S
34	S	S	S	S	S	S
35	R	R	R	I	R	S
36	I	R	R	R	R	S
37	S	S	R	R	R	S
38	R	R	R	R	R	S
39	R	R	R	R	R	S
40	R	R	R	R	R	S
41	I	R	R	R	R	S
42	S	S	S	S	S	S
43	S	S	R	S	S	S
44	S	S	S	S	S	S
45	S	S	S	S	S	S
46	R	R	R	R	R	S
47	S	S	S	S	S	S
48	S	S	R	S	S	S
49	S	S	R	S	S	S
50	R	R	R	R	R	S
51	S	S	R	S	S	S
52	I	I	R	I	R	S
%Susceptibility	**61.5**	**61.5**	**~30.8**	**~46.2**	**~53.9**	**100.0**
%Intermediate	**11.5**	**3.8**	**3.8**	**19.2**	**3.8**	**0.0**
%Resistance	**~ 27.0**	**34.6**	**~65.4**	**34.6**	**42.3**	**0.0**

The results obtained from new 26 strains collected during 2017 period showed a new prevalence for all tested antibiotics such as penicillin (27.0%), ampicillin (34.6%), oxacillin (65.4%), amoxicillin plus clavulanic acid (34.6%) and cefazolin (42.3%). However, an intermediate resistant rates of 11.5%, 3.8%, 3.8%, 19.2% and 3.8% were found for these already described antibiotics, respectively. Finally, piperacillin antibiotic showed forever a suscptebility of 100.0% against all these new collected strains **(Table 15)**. The comparison of these new findings with those mainly found in 2003-2005 and 2007-2008 periods are widly different. As a result, we observed a noticeable reduction especially for penicillin (27.0% in 2017 instead of 76.9% in 2003 to 2008 period) and for ampicillin (34.6% in 2017 instead of 73.0% in 2003 to 2008 period). This noted decrease in prevalence of both antibotics penicillin and ampicillin in

2017 could be associated to veterinarian practices; for example, because of high resistance prevalence emerged of *S. aureus* against penicicillin and ampicillin in the world had lead many veterinarians to avoid the use and prescription of these well-known antibiotics in treatment of bacterial infections especially mastitis disease caused by *S. aureus* in view of presence of a bad reputation that marked the Beta-lactams family worldly. As a result of this, we can conclude that this noted reduction in prevalence of both antiotics penicillin and ampicillin could be explained by inactivation of genes (such as *blaZ*, *mecA*) responsible genes encoded for resistance phenomenon to both prominent antibiotics. However, the new strains showed a resistant prevalence of (65.4% in 2017 instead of 3.8% in 2003 to 2008), (34.6% in 2017 instead of 0.0% in 2003 to 2008) and (42.3% in 2017 instead of 0.0% in 2003 to 2008) for oxacillin, amoxicillin associate with clavulanic acid and cefazolin, prespectivly. On the based data, the manifestation of intermediate resistant strains must be taken into consideration; because, this phenomenon could be associated to development of genes encoded for these antibiotics such as amoxicillin associate with clavulanic acid, cefazolin on the one hand or to progressive inactivation of these genes by no use of bacteria for these genes for several years what make them unexpressed. Finally, piperacillin antibiotic saved its effectiveness against older strains collected in 2003 to 2008, and it is also found to be very effective against new strains that have been recently collected in 2017. What needs to reconsider this antibiotic in order to fight and treat *S. aueus* infections originated from bovine mastitis.

Table 16: Antibiotic resistance genes profile (*blaZ* and *mecA*) of the tested *S. aureus* isolates from bovine mastitis by PCR during 2017 period.

Genes	Isolates tested																										%
	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	
blaZ	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	**34.6**
mecA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0.0**

According to PCR results, 9 among 26 *S. aureus* new strains (34.6%) retrieved from mastitis milk samples during 2017 year carrying *blaZ* gene that was performed through successful amplification of (377bp) specific products, and all these strains are negative for *mecA* gene (**Table 16**). In general, these genotype findings are in most cases in correspondence with what we found for phenotype findings especially for both antibiotics penicillin and ampecillin, except for isolates N° 27, 28 and 37 that were the only positives for *blaZ* gene while they were

susceptible against these both antiotics. This could be associated also to the non expression of *blaZ* gene but we can consider these three strains as potentially resistant. Concerning the results obtained from susceptibility testing profiles to oxacillin and amoxicillin associate with clavulanic acid antibiotics were mostly in correspondence with results obtained for genotypic testing profile for *blaZ* gene, except for isolates N° 29, 46 and 52 for both antibiotics oxacillin and amoxicillin associate to clavulanic acid. Thus, the resistant observed to these three strains could be attributable to presence of other genes encoding for these drugs such as *mecC* that was described for the first time in the 2011. *mecC* (formerly *mecA*LGA251) is also a Beta-lactams resistance gene, but is less well understood than *mecA* gene.

In the final of this first part of this study focused on ''Beta-lactam antimicrobials activity and the diversity of *blaZ* gene in *Staphylococcus aureus* isolates from bovine mastitis in the Northwest of Portugal'', we have grouped our results from 2003 to 2017 in order to have a global vision on the resistance profiles of *S. aureus* originated from bovine mastitis in the northern west of Portugal. In general, the results obtained from the antimicrobial susceptibility testing for all the tested 52 *S. aureus* isolates showed a prevalence of 57.7% (n=30) (R=52.0%, n=27/52; I=5.7%, n=3/52) of resistant strains to penicillin followed by a prevalence of 53.9% (n=28) (R=52.%, n=27/52; I=1.9%, n=1/52) of resistance to ampicillin and a prevalence of 36.5% (n=19) (R=34.6%, n=18/52; I=1.9%, n=1/52), 26.9% (n=14) (R=17.3%, n=9/52; I=9.6%, n=5/52) and 23% (n=12) (R=21.1%, n=11/52; I=1.9%, n=1/52) of resistance to oxacillin, amoxicillin associate with clavulanic acid and to cefazolin, respectively. A very high susceptibility prevalence (100.0%, n=52) was demonstrated for piperacillin (**Table 17**). However, the results obtained for the *blaZ* and *mecA* resistance genes profiles showed that among all tested *S. aureus* isolates (n=52), 67.3% (n=35) were PCR positive for *blaZ* and negative for *mecA* genes, except for isolate 25 that was the only one positive for *mecA* (**Table 18**). In addition, the phylogenetic analysis of sequences of *blaZ* region and the relationship between the phenotypic and genotypic antibiotic profile of the tested *S. aureus* isolates during 2003 to 2017 have been also studied (**Table 19, Table 20** and **Figure 45**). Of note, more results are presented in the following tables and figures figured bellow while more details and data are given and described in discussion axis.

Table 17: Antimicrobial tests performed to all *S. aureus* isolates from bovine mastitis against β-lactam antibiotics by disk diffusion method following CLSI guidelines.

Isolates	β-lactam antibiotics in disks					
	PEN(10 U)	**AMP (10 µg)**	**OXA(1 µg)**	**AMC (20 µg +10 µg)**	**CFZ (30 µg)**	**PIP (100 µg)**
1	R	R	S	S	S	S
2	R	R	S	S	S	S
3	R	R	S	S	S	S
4	R	R	S	S	S	S
5	R	R	S	S	S	S
6	R	R	S	S	S	S
7	R	R	S	S	S	S
8	R	R	S	S	S	S
9	R	R	S	S	S	S
10	S	S	S	S	S	S
11	R	R	S	S	S	S
12	R	R	S	S	S	S
13	R	R	S	S	S	S
14	R	R	S	S	S	S
15	R	R	S	S	S	S
16	R	R	S	S	S	S
17	R	R	S	S	S	S
18	R	S	S	S	S	S
19	S	S	S	S	S	S
20	R	R	S	S	S	S
21	S	S	S	S	S	S
22	S	S	S	S	S	S
23	S	S	S	S	S	S
24	R	R	S	S	S	S
25	R	R	R	S	S	S
26	S	S	S	S	S	S
27	S	S	R	I	I	S
28	S	S	I	I	S	S
29	R	R	R	R	R	S
30	S	S	S	S	S	S
31	S	S	S	S	S	S
32	S	S	S	S	S	S
33	S	S	R	I	S	S
34	S	S	S	S	S	S
35	R	R	R	I	R	S
36	I	R	R	R	R	S
37	S	S	R	R	R	S
38	R	R	R	R	R	S
39	R	R	R	R	R	S
40	R	R	R	R	R	S
41	I	R	R	R	R	S
42	S	S	S	S	S	S
43	S	S	R	S	S	S
44	S	S	S	S	S	S
45	S	S	S	S	S	S
46	R	R	R	R	R	S
47	S	S	S	S	S	S
48	S	S	R	S	S	S
49	S	S	R	S	S	S
50	R	R	R	R	R	S
51	S	S	R	S	S	S
52	I	I	R	I	R	S
%Susceptibility	**~42.3**	**~46.1**	**~63.5**	**~73.1**	**~77**	**100.0**
%Intermediate	**5.7**	**1.9**	**1.9**	**9.6**	**1.9**	**0.0**
%Resistance	**52**	**52**	**34.6**	**17.3**	**21.1**	**0.0**

Concerning the results obtained for the *blaZ* and *mecA* resistance genes profiles. Among all tested *S. aureus* isolates (n=52), 67.3% (n=35) were PCR positive for *blaZ* and negative for *mecA* genes, except for isolate 25 that was the only one positive for *mecA* (**Table 18**).

Table 18: PCRs tests performed to all 52 *S. aureus* isolates from bovine mastitis.

No. Herd	Year of samples collection	Nº Isolate	*BlaZ* gene	*mecA* gene
1	2003	1	Positive	Negative
	2008	2	Positive	Negative
	2008	3	Positive	Negative
2	2003	4	Positive	Negative
3	2004	5	Positive	Negative
4	2003	6	Positive	Negative
5	2003	7	Positive	Negative
6	2003	8	Positive	Negative
7	2004	9	Positive	Negative
8	2003	10	Positive	Negative
	2003	11	Positive	Negative
9	2003	12	Positive	Negative
10	2003	13	Positive	Negative
11	2003	14	Positive	Negative
12	2003	15	Positive	Negative
13	2003	16	Positive	Negative
14	2003	17	Positive	Negative
15	2003	18	Positive	Negative
16	2004	19	Positive	Negative
17	2007	20	Positive	Negative
18	2008	21	Positive	Negative
19	2008	22	Positive	Negative
20	2008	23	Positive	Negative
	2008	24	Positive	Negative
21	2008	25	Positive	Positive
22	2008	26	Positive	Negative
23	2017	27	Positive	Negative
	2017	28	Positive	Negative
	2017	29	Negative	Negative
24	2017	30	Negative	Negative
	2017	31	Negative	Negative
25	2017	32	Negative	Negative
	2017	33	Negative	Negative
26	2017	34	Negative	Negative
27	2017	35	Positive	Negative
28	2017	36	Positive	Negative
29	2017	37	Positive	Negative
	2017	38	Positive	Negative
	2017	39	Positive	Negative
	2017	40	Positive	Negative
30	2017	41	Negative	Negative
	2017	42	Negative	Negative
31	2017	43	Negative	Negative
	2017	44	Negative	Negative
	2017	45	Negative	Negative
32	2017	46	Negative	Negative
33	2017	47	Negative	Negative
34	2017	48	Negative	Negative
35	2017	49	Negative	Negative
36	2017	50	Positive	Negative
37	2017	51	Negative	Negative
	2017	52	Negative	Negative
Resistance%	**-**	**-**	**67.3%**	**1.9%**

Table 19: Relationship between the phenotypic and genotypic antibiotic profile of the tested *S. aureus* isolates during 2003 to 2017.

Number Of isolates	B-lactam antibiotics (µg in disk)						Resistance Genes	
	PEN(6)	AMP(10)	OXA(1)	AMC(20+10)	CFZ(30)	**PIP(100)**	BlaZ	MecA
1	R	R	S	S	S	**S**	+	-
2	**S**	**S**	**S**	**S**	**S**	**S**	+	-
3	R	R	S	S	S	**S**	+	-
4	R	R	S	S	S	**S**	+	-
5	R	R	S	S	S	**S**	+	-
6	R	R	S	S	S	**S**	+	-
7	R	R	S	S	S	**S**	+	-
8	R	R	S	S	S	**S**	+	-
9	R	R	S	S	S	**S**	+	-
10	R	R	S	S	S	**S**	+	-
11	R	R	S	S	S	**S**	+	-
12	**S**	**S**	**S**	**S**	**S**	**S**	+	-
13	R	R	S	S	S	**S**	+	-
14	R	R	S	S	S	**S**	+	-
15	R	R	S	S	S	**S**	+	-
16	R	R	S	S	S	**S**	+	-
17	R	R	S	S	S	**S**	+	-
18	R	R	S	S	S	**S**	+	-
19	R	R	S	S	S	**S**	+	-
20	R	S	S	S	S	**S**	+	-
21	**S**	**S**	**S**	**S**	**S**	**S**	+	-
22	R	R	S	S	S	**S**	+	-
23	**S**	**S**	**S**	**S**	**S**	**S**	+	-
24	**S**	**S**	**S**	**S**	**S**	**S**	+	-
25	R	R	R	R	S	**S**	+	+
26	**S**	**S**	**S**	**S**	**S**	**S**	+	-
27	S	S	R	I	I	**S**	+	-
28	S	S	I	I	S	**S**	+	-
29	R	R	R	R	R	**S**	-	-
30	S	S	S	S	S	**S**	-	-
31	S	S	S	S	S	**S**	-	-
32	S	S	S	S	S	**S**	-	-
33	S	S	R	I	S	**S**	-	-
34	S	S	S	S	S	**S**	-	-
35	R	R	R	I	R	**S**	+	-
36	I	R	R	R	R	**S**	+	-
37	S	S	R	R	R	**S**	+	-
38	R	R	R	R	R	**S**	+	-
39	R	R	R	R	R	**S**	+	-
40	R	R	R	R	R	**S**	+	-
41	I	R	R	R	R	**S**	-	-
42	S	S	S	S	S	**S**	-	-
43	S	S	R	S	S	**S**	-	-
44	S	S	S	S	S	**S**	-	-
45	S	S	S	S	S	**S**	-	-
46	R	R	R	R	R	**S**	-	-
47	S	S	S	S	S	**S**	-	-
48	S	S	R	S	S	**S**	-	-
49	S	S	R	S	S	**S**	-	-
50	R	R	R	R	R	**S**	+	-
51	S	S	R	S	S	**S**	-	-
52	I	I	R	I	R	**S**	-	-

Sequencing analysis of *blaZ*

Thirty-two positive isolates were selected for sequencing partial *blaZ* gene. The retrieved sequences were analyzed and a consensus sequence for each isolate was created. When blastn of nucleotide consensus sequences were conducted in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), similarities of 100.0-99.0% and e-values of 0.0-5e-158 were shared for *S. aureus* strains (**Table 20**). This data supported the selection of the *S. aureus* strains used in the phylogenetic analysis.

Table 20: Blastn between consensus sequences of all 52 tested bovine mastitis *S. aureus* isolates in the NCBI-GenBank database. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Isolate	GenBank Acession no.	Strain	Position	E-value	% Identities
1	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008879	0.0	100.0
2	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
3	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008865	0.0	100.0
4	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
5	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 – 2008879	0.0	99.0
6	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
7	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
8	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
9	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
10	DQ016066	*Staphylococcus aureus* strain A1358_1	181 – 544	0.0	100.0
11	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
12	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
13	DQ016066	*Staphylococcus aureus* strain A1358_1	181 – 543	0.0	100.0
14	CP011528	*Staphylococcus aureus* strain RKI4	1929299 – 1929676	0.0	99.0
15	CP012971	*Staphylococcus aureus* strain ST20130939	13433 – 13797	0.0	99.0
16	CP012971	*Staphylococcus aureus* strain ST20130939	13433 – 13797	0.0	99.0
17	CP012971	*Staphylococcus aureus* strain ST20130939	13420 – 13797	0.0	99.0
18	CP012971	*Staphylococcus aureus* strain ST20130939	13420 – 13797	0.0	99.0
19	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
20	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
21	CP011528	*Staphylococcus aureus* strain RKI4	1929299 – 1929676	0.0	99.0
22	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
23	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
24	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	99.0
25	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
26	DQ016066	*Staphylococcus aureus* strain A1358_1	168 – 544	0.0	100.0
36	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0
38	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0
39	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0
40	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0
41	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0
50	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 – 2886572	5,00E-158	100.0

Phylogenetic analysis of *blaZ*

Phylogenetic relationships were inferred using the ML method as described in methods section. The phylogenetic analysis placed the 32 *S. aureus* isolates in 2 different clades, clade A and B, supported by bootstrap values of 76.0% and 93.0% (of 1.000 replicates) respectively (**Figure 45**). The isolates 1, 3-13, 19-20 and 22-26 were placed in clade A, supported by bootstrap values of 76.0% (of 1.000 replicates), and are closely related to bovine and human *S. aureus* strains (**Figure 45**). Moreover, within clade A there is a cluster A with a sub-cluster A.1, supported by bootstrap values of 85.0% and 73.0% (of 1.000 replicates) respectively. Isolate 21 is placed as single within cluster A, being the most divergent within cluster A and isolate 14 is placed in the sub-cluster A.1 closely related to a human *S. aureus* strain (**Figure 45**). Relatively to clade B, isolates 15-18 are placed more closely related to human *S. aureus* strains, and within cluster B isolates 36, 38-41 and 50 appeared placed more closely related to animal food, animal and human *S. aureus* strains (**Figure 45**). Lastly, isolate 2 appeared in the phylogenetic tree as the most divergent of all analyzed *S. aureus* strains in this study (**Figure 45**).

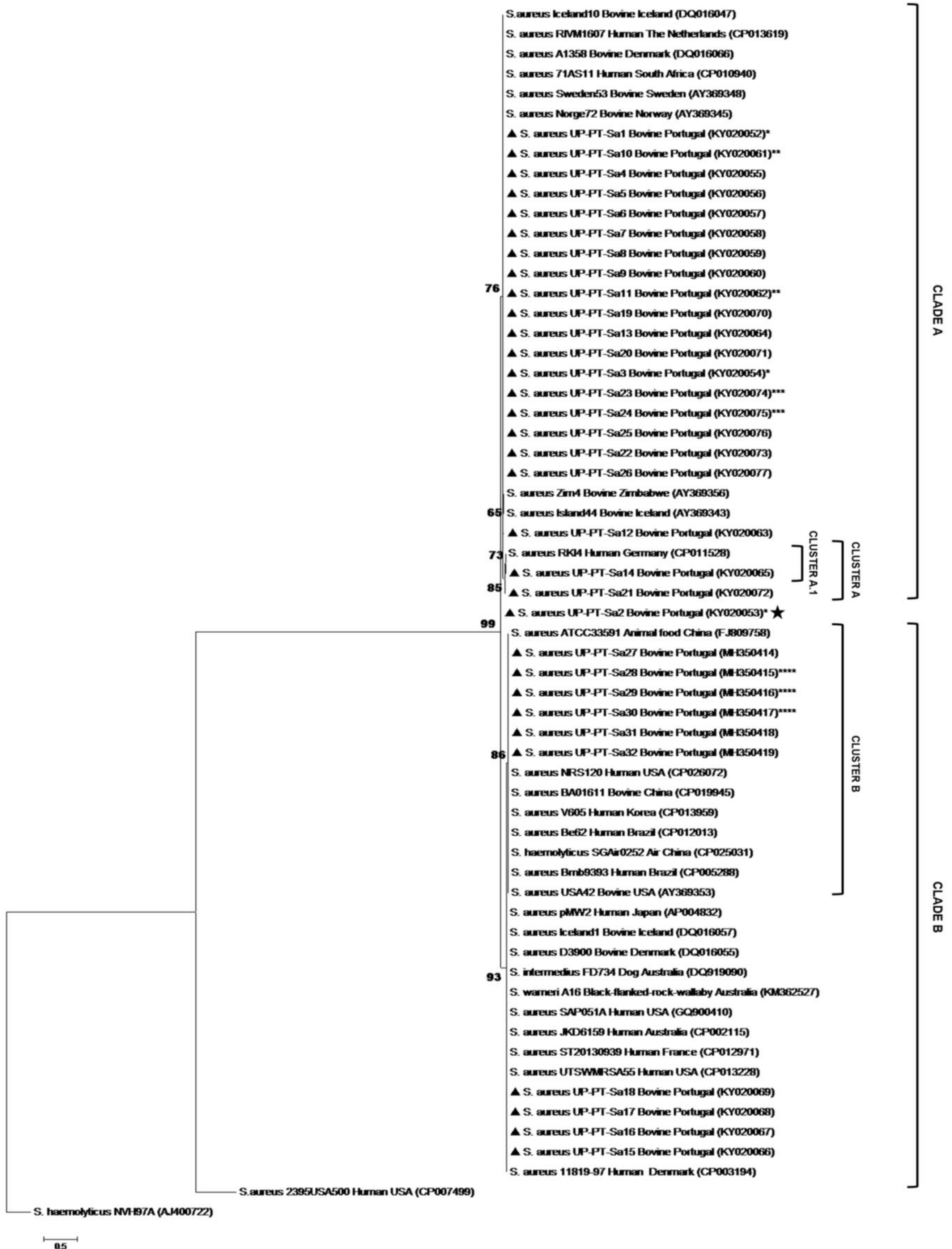

Figure 45: Phylogenetic analysis of sequences of *blaZ* region in bovine mastitis *S. aureus* isolates. ML method was inferred. Bootstrap resampling was used to determine the robustness of branches; values from 1,000 replicates are shown. Filled triangle, indicates the 52 bovine mastitis *S. aureus* isolates tested in this study.

The new results obtained from the antimicrobial susceptibility testing for all isolates, collected in years 2003-2004, 2007-2008 and 2017 in the northwest of Portugal, showed a resistance prevalence of 57.7% (R=52%, n=27/52; I=5.7%, n=3/52) to penicillin (Penicillin G group), followed by 53.9% to ampicillin (R=52%, n=27/52; I=1.9%, n=1/52) (Aminopenicillin group) and 36.5% (R=34.6%, n=18/52; I=1.9%, n=1/52), 26.9% (R=17.3%, n=9/52; I=9.6%, n=5/52) and 23% (R=21.1%, n=11/52; I=1.9%, n=1/52) for oxacillin (Penicillin M group), amoxicillin plus clavulanic acid (Penicillin M group) and for cefazolin (Cephalosporin group), respectively. Other antimicrobial included in the study within β-lactams groups, namely piperacillin (Ureidopenicillin group) showed always 100.0% of *in vitro* activity against tested *S. aureus* isolates. Although the studies on resistance by *S. aureus* causing bovine mastitis in Portugal are scarse, the trends in antibacterial resistance of major bovine mastitis pathogens were reported to have improved in dairy herds in the northwestern, central and southern regions of Portugal (**Rocha *et al.,* 2014**). However, a moderate high prevalence of resistance to penicillin (66.7%, n=20) was described in the central region of Portugal (**Nunes *et al.,* 2007**) when compared to that found in this study (57.7%) (R=52%, n=27/52; I=5.7%, n=3/52). The resistance against β-lactam antibiotics especially penicillin, ampicillin and amoxicillin were described in numerous different geographical regions, such as in the European, African and Latin America countries. For example, in Estonia and Finland dairy herds, a prevalence resistance to bovine mastitis *S. aureus* strains was described to round 61.4 and 52.1% respectively (**Pitkala *et al.,* 2004; Kalmus *et al.,* 2011**). Furthermore, a prevalence resistance to bovine mastitis *S. aureus* strains for amoxicillin, ampicillin and penicillin of 20.6 and 36.0% was described in New Zealand and in the USA, respectively (**Petrovski *et al.,* 2015**). The overall findings in the patterns of resistance are less high or much lower than the obtained in our study. These differences could be associated to the drugs selection used for treatment in each country, as the choice of antimicrobials to be applied will depend on the local availability and respective regulations. In Lithuania, **Klimienė *et al.* (2011)**, showed different rates of penicillin resistance (76.7%) with a high increased level for ampicillin (78.4%) and amoxicillin (81.3%) in comparison to our study, suggesting that amoxicillin appeared less effective against bovine mastitis by *S. aureus* strains in Lithuania than in Portuguese dairy herds. Studies in Africa (South West Ethiopia) (**Sori *et al.,* 2011**), showed that isolates had a high resistance prevalence (87.2%) to penicillin in comparison to those found in our study, achieving in the contrary, a higher resistance prevalence (46.0%) for amoxicillin alone than we found for amoxicillin associate to clavulanic acid (26.9%) (R=17.3%, n=9/52; I=9.6%, n=5/52). When comparing our results with those described in Latin American countries, we found a high prevalence rate of resistance to

penicillin (57.7%) (R=52%, n=27/52; I=5.7%, n=3/52) than the described in Argentinian dairy herds (48.4%). Moreover, the resistance prevalence for oxacillin obtained in our study was of 36.5% (R=34.6%, n=18/52; I=1.9%, n=1/52) while they described 0.0% (**Russi *et al.,* 2008**). Although, a phenotypic resistance to oxacillin of 12.8% has been previously described in *Staphylococcus* spp including *S. aureus* isolated from small ruminant's mastitis in Brazil (**França *et al.,* 2012**).

For the remaining groups of antibiotics tested within the Beta-lactams family, namely piperacillin showed a complete (100.0%) activity against all tested bovine mastitis *S. aureus* isolates. There are few studies focusing this antimicrobial. In 2015, **Sharma *et al.,* (2015)** reported a complete (100.0%) antimicrobial sensitivity to *S. aureus* isolates obtained from clinical and sub-clinical mastitis against imipenem and a high antimicrobial sensitivity (88.9%) against piperacillin plus tazobactam, however an antimicrobial resistance of 33.3% was described for cefazolin, higher than what we observed here (23.0%).

The detection of *blaZ* and *mecA* resistance genes showed that among all tested bovine mastitis *S. aureus* isolates, 67.3% of them were positive for the *blaZ* and negative for *mecA* resistance genes, except for isolate 25 that was positive for *mecA*. The Beta-lactam antibiotics tested in this study and the antimicrobial resistance found within *S. aureus* isolates suggests a direct link to *blaZ* gene. Interestingly, isolate 25, positive for *mecA* gene, was found to be resistant to oxacillin, the antibiotic that is nowadays used instead of methicillin because of it is less toxic anti-staphylococcal penicillins **(Cunha, 2005)**. Therefore, oxacillin antibiotic became the antibiotic used to detect methicillin resistance **(Cunha, 2005)**. The resistance of *S. aureus* to oxacillin due to the acquisition of the *mecA* gene has been previously described (**Al-Akydy *et al.,* 2014**). There are numerous mechanisms of antimicrobial resistance to Beta-lactam antibiotics and one of the most important is associated with the production of Beta-lactamases (**Bush *et al.,* 1995; Mcmanus, 1997; Keith *et al.,* 2000**). While in a specific region, other factors can be involved in the resistance against a particular antibiotic, it can be due to the frequent and long-term antibiotic utilization (**Sabour *et al.,* 2004; Moon *et al.,* 2007; Kumar R *et al.,* 2010; Sharma *et al.,* 2015**). In other published studies on this topic, it appeared that the prevalence of resistance to antibiotics (penicillin and ampicillin) is higher in bovine mastitis caused by *S. aureus* (**Li *et al.,* 2009**). Furthermore, in most cases, authors have associated the high rates of resistance encountered in bovine mastitis *S. aureus* strains with the production of β-lactamase encoded by the gene *blaZ* (**Watts & Salmon, 1997; Szweda *et al.,* 2014**).

In general, the penicillin resistance presented by *S. aureus* is conferred by two well-known mechanisms. One mechanism of resistance to penicillin, considered to be the most important,

is directly associated with the expression of the enzyme β-lactamase, which can hydrolyze the antibiotic so rendering it inactive (**Hartman & Tomasz, 1984**). The second, primarily related with human isolates, is responsible to the resistance linked to the PBP2a protein, encoded by *mecA* gene, and plays a role in methicillin resistance, which is a much less sensitive target than the wild-type PBPs (**Hartman & Tomasz, 1984; Deurenberg *et al.,* 2007**).

In the present study, there are 6 *S. aureus* isolates (2, 12, 21, 23, 24, 26) with phenotypic susceptibility against all tested antibiotics that also harbor *blaZ* resistance gene (**Table 17 and Table 19**). To our knowledge, there is not enough data explaining this phenomenon, but **Haveri *et al.* (2005)** have suggested that phenotypically susceptible isolates that carry resistance genes should be considered as potentially resistant. Our suggestion to explain this phenomenon, could be for example that, the 6 cows could have been administrated with other antibiotics of the β-lactam family in place of conventional antibiotics (such as penicillin and ampicillin), or that the presence of a mutation or a set of mutations could have occurred at the level of the gene encoded for this resistance. Furthermore, besides detecting the gene, further studies on the evaluation of the gene expression/unexpression must be done to better explain this phenomenon. Recent data reported by **Tasara *et al.* (2013)** showed that among 10 *S. aureus* strains carrying *blaZ* gene, 5 strains were phenotypically resistant to penicillin while the other 5 (all belonging the clonal complex 8) were susceptible to penicillin (**Tasara *et al*., 2013**). The presence of the *blaZ* in all 5 strains were confirmed by PCR, while the sequencing results of these genes uncovered a 29 base deletion within the *blaZ* gene in all these strains that cause a translational frame shift, which is predicted to induce abrogation of *blaZ* expression (**Tasara *et al*., 2013**). On the other hand, the possibility of incorrect procedure in the antimicrobial testing performance was out of question, as all tests were performed equally and with achieved repeatable different results. Here, different sequences were recovered from the 9 *S. aureus* tested isolates and phylogenetically they were placed in different clusters, disrupting the possibility of contamination during the *blaZ* PCR procedure.

The *blaZ* gene phylogenetic analysis placed the 32 *S. aureus* isolates selected for sequencing in 2 different clades, clade A and B, and they are closely related to different bovine mastitis and/or human *S. aureus* strains. The study involved isolates from bovine mastitis samples collected in the years of 2003, 2004, 2007, 2008 and 2017 in herds geographically nearby**,** and as expected there was a phylogenetic divergence of analyzed strains observed during this period of time. Almost all isolates belonging to the years of collection 2003, 2004, 2007 and 2008 are placed in the clade A, which appeared closely related to animal and human *S. aureus* strains. Although, isolates 15, 16, 17 and 18 (2003) appeared placed in the clade B

with the recent (2017) *S. aureus* isolates, all also related with animal and human *S. aureus* strains. To note, and as expected, the recent isolates appeared phylogenetic separately from the 2003 *S. aureus* strains in the clade B, being genetically related between them, but phylogenetically divergent from the 2003 *S. aureus* strains. Furthermore, isolate 2 (collected in 2003, from herd 1 as isolates 1 (2003) and 3 (2008)), appeared placed single in the phylogenetic tree, being the most phylogenetically divergent strain in this study, and, even though it is a "relatively older" *S. aureus* strain, maybe it will be interesting to deepen it genetically in the future. In addition, it is also relevant to refer that the older isolates in this study (mainly placed in clade A) are related to a high prevalence of penicillin and ampicillin resistance, and that the recently ones (placed in clade B) are related with a higher prevalence of resistance to almost all tested antibiotics (penicillin, ampicillin, oxacillin, amoxicillin plus clavulanic acid and cefazolin), suggesting that this is in the line of evolution/divergence of the strains in the testing period of time, by the use, or the excess use of antibiotics in the animal treatments.

We were able to detect and sequence *blaZ* gene according to the methods previously described (**Olsen *et al.,* 2006**), however, the evaluated diversity in this study, as described above, was not done in the context of chromosome or plasmid gene location. As well, we were able to reveal and elucidate the management of the use of antibiotics in mastitis as we were also able to detect and sequence *blaZ* gene according to the methods previously described (**Olsen *et al.,* 2006**).

In conclusion, this study indicates that *blaZ* resistance gene plays a role in β-lactam resistance in the tested bovine mastitis *S. aureus* isolates within dairy herds in the north of Portugal, especially in case of penicillin and ampicillin antibiotics that have shown a high phenotypic prevalence. However, the proportion of bovine mastitis isolates with phenotypic resistance did not agree with the proportion of those identified with *blaZ*, as isolates with 100.0% of phenotypic susceptibility for all tested antibiotics also harbored *blaZ.* The antibiotic piperacillin showed high *in vitro* activity against *S. aureus* isolates, suggesting that this could be chosen for *in vivo* treatments instead of penicillin or ampicillin, which is frequently used. Finally, *blaZ* phylogenetic analysis from *S. aureus* isolates showed diversity inside or between different herds in the northwest of Portugal. The evaluation of new bovine mastitis milk samples collected in the same herds, using the same or other methods would be of importance to further discuss the dynamics on resistance patterns of *S. aureus* in the region.

In the first part (I) of this thesis, we have grouped our results from 2003 to 2017 in order to give a global vision on the resistance profiles of *S. aureus* against a set of antibiotics group from Beta-lactam family. However; in the second part (II), we have extended our analysis to other antibiotics such as gentamicin, tobramycin, amikacin and kanamycin, all from Aminosides family; tetracycline from Tetracycline family; erythromycin and clindamycin from Macrolides family; vancomycin from Glycopeptides family and other antibiotics class such as trimethoprim/sulfamethoxazole from lipopeptides family in order to have also a vision on the resistance profiles of *S. aureus* against these antibiotics from different families more used in veterinary medicine.

Worldly, Beta-lactams family are considered as the most widely described and used antibiotics in clinical medicine and resistance to these prominent medicines may become a severe threat because they have low toxicity and are used to treat a broad range of infections (**Livermore, 1996; Aminov, 2009**). The begning introduction of antibiotics, especially penicillin the first Beta-lactams antibiotics, into clinical practice in the 1940s led to save many millions of lives and placed the majority of infectious diseases that plagued human history for many centuries under control. Really, antibiotics were extremely effective in clearing pathogenic bacteria leading many to believe that infectious diseases would become a problem of the past and would be wiped out from all human populations eventually (**Aminov, 2010**). At that time, almost of infections caused by *Staphylococcae* species can be easily treated with these new discovered antibiotics. Thus, penicillin has been the drug of choice for treatment of infections caused by *S. aureus*; but, the emergence of resistant *S. aureus* strains to penicillin were unfortunately reported as early as 1944 (**Kirby, 1944**). In the recent years, the prevalence of human penicillin resistant *S. aureus* reached around 90% while the prevalence of penicillin-resistant bovine isolates varies from 10 to 70% in relation to geographic location (**Olsen *et al.,* 2006**). Methicillin is β-lactam antibiotic invented in order to treat penicillin-resistant *S. aureus*; but, the first MRSA was soon reported 2 years after the introduction of methicillin in clinical medicine in 1961 (**DeLeo & Chambers, 2009; Simonetti *et al.,* 2011; Kejela & Bacha, 2013**). The successful deployment of antibiotics has unfortunately resulted in these drugs being used more as a financial commodity rather than a valuable community resource that should be rationally managed. This has conducted to the accelerated development of antimicrobial resistance among many bacteria over the world (**Lewies *et al.,* 2019**).

The use of antimicrobial agents with narrow broad-specrum in addition to the use of combined antibiotics remained among the new successful strategies followed in the last decades for more effectivness of these agents against several resistant pathogenic bacteria especially *S. aureus*

causing many serious infectious diseases; but, all these strategies, along with time, showed their limited effectiveness and stayed as temporary solutions in the light of progressive emergence of multidrug-resistant strains. Over decades, many antimicrobial agents families with different mechanisms of action on microorganisms have been parallely introduced in order to overcome the resistance problem. For example, family of Aminoglycosides, Tetracyclines, Macrolides Lincosamides, Streptogramins, Glycopeptides, Lipopeptides, ... etc. These antibiotics families are extensively used and more described in veterinary medicine for cost-effective prophylactic and therapeutic treatment and they are also used as growth promoters in cattle and poultry. Despite the many efforts deployed in this regards, the majority of *S. aureus* strains are nowdays became multidrugs-resistance because of acquisition of several resistance genes encoding for almost of antimicrobials agents.

In the literature reports, the pathogenic resistance bacteria, particularly *S. aureus,* have nowadays developed and acquired several mechanisms allowing them to avoid efficacy of antimicrobials. For example, many genes such as *blaZ*, *mec*, *erm*, *aac/aph*, *tet*, *vga* and *van* are among the prevalent resistance genes noted to play a role in *S. aureus* resistance encoded for several antibiotics such as Beta-lactams, Macrolides, Aminoglycosides, Tetracyclines and Glycopeptides families (**Weisblum, 1995; Vahaboglu *et al.,* 1998; Lina *et al.,* 1999; Martineau *et al.,* 2000; Strommenger *et al.,* 2003; Choi *et al.,* 2003; DeLeo & Chambers, 2009; Fabler *et al.,* 2010; Nizami *et al.,* 2012; Tang *et al.,* 2015; Farid *et al.,* 2015; Pekana & Green, 2018; Ma *et al.,* 2018**).

Part II:

Genotypic and Phenotypic Characterization of *Staphylococcus aureus* Isolated from Bovine Mastitis in the Northwest of Portugal

Abstract

Staphylococcus aureus (*S. aureus*) is considered as one of the most widespread microorganisms associated with infections among humans and animals and has shown a frequent and rapid development of antibiotic resistance.

Objective: The aim of the present study was to investigate the prevalence, evaluate in vitro efficacy of a set of antibiotics to the resistance phenomenon and related resistance genes among *S. aureus* isolated from bovine mastitis in the northwest of Portugal.

Methods: The antimicrobial susceptibility tests were performed by the disk diffusion method. The detection of genes such as aminoglycoside-modifying enzymes (*aph(3')-III-a, ant(4)-I-a and aac (6') -aph(2')*), *tetM*, *tetK*, *ermT*, *vanA*, *spc*, *lnuC*, *salA*, *vgaC* and *dfrK*, was performed using specific PCRs methods.

Results: Of the 52 *S. aureus* isolates, 39 (75.0%), 35 (67.3%), 26 (50.0%), 22 (42.3%), 19 (36.5%), 17 (32.7%), 15 (28.8%), 12 (23.1%) and 11 (21.2%) showed high resistance to tetracycline, erythromycin, vancomycin, amikacine, kanamycin, clindamycin, gentamycin, tobramycin and trimetroprim-sulfatomexazole, respectively. On the other hand, 18 (34.0%), 15 (28.8%), 14 (26.9%), 11 (21.2%), 7 (13.5% each), and 4 (7.7% each) showed intermediate resistance to vancomycin, trimetroprim-sulfatomexazole, tobramycin, gentamycin, tetracycline, erythromycin, kanamycin, clindamycin and amikacin, respectively. Of the screening genes, *ant(4)-I-a*, *tetM*, *aph(3')-II I-a*, *dfrK*, *tetK*, *lnuC* and *spc* were detected in 33 (63.5%), 30 (57.7%), 16 (30.8%), 10 (19.2%), 8 (15.4%), 3 (5.8%) and 1 (1.9%), respectively. Moreover, the remaining tested genes such as *vanA*, *ermT*, *salA*, *vgaC*, *aac (6') - aph (2')* were all found negatives. All of the antibiotics used in this study showed a high efficacy towards all the strains collected during the period from 2003 to 2008. Whilst, all the new strains collected more recently in 2017 were in most cases either resistant or intermediate against all these antibiotics; thus, showing an alarming and dramatic evolution of appearance of new very

resistant strains leading to think immediately to permanently change our habitual vision and reaction to each use of these antibiotics before that humanity declares a total capitulation to new infections caused by *S. aureus* in few coming years.

In conclusion of this second part, our results illustrated the presence of a high prevalence more than 50.0% of phenotypic resistance was found in the tested *S. aureus* isolates against tetracycline, erythromycin and also for vancomycin. Moreover, a high prevalence of 63.5% and 57.7% was detected, by using PCR, for *ant(4)-I-a and tetM,* respectively. The findings presented from this study could be advantageous for designing specific programs of control for bovine mastitis disease caused by *S. aureus* in the northwest region of Portugal.

Introduction

In veterinary and human medicine, antimicrobial agents play an important role in the therapy of bacterial infections (**Wendlandt *et al*., 2015**). But, the resistance problem that human and animal health encountered often after use of these antimicrobial agents against these bacterial infections manifest, often quickly, after each introduction of new classes of antimicrobial drugs (**Knobler *et al*., 2003**). The discovery of antimicrobial agents during the 20th century has always been considered one of the wonder discoveries in that time (**Davies & Davies, 2010**) but the veritable wonder is the increase of antimicrobials resistance encountered in hospitals, communities, and the environment concomitant with their utilization (**Davies & Davies, 2010**). Antimicrobials are used for treatment of a variety of diseases and of about 80% of conventional dairy herds reported the utilization of antibiotics in order to treat mastitis (**Zwald *et al*., 2004**). The first antimicrobials usage in veterinary medicine was noted in dairy cows for treatment of mastitis disease (**Mitchell *et al*., 1998**). Worldwide, bovine mastitis is considered to be a common disease touching dairy cows with high incidence (**Gomes *et al*., 2016**), most expensive to the dairy industry (**Gomes *et al*., 2016**) and also one of the most considerable factors limiting profitability of dairy farm (**Ruegg *et al*., 2015**) as it's the single most common reason for antimicrobial drug utilization in dairy herds, because antibiotic therapy remains a primary tool and a major component for mastitis control in lactating and dry cows (**Rajala-Schultz *et al*., 2004**). The appearance of mastitis bovine can be in a clinical and subclinical form, the latter form being frequently encountered in most herds (**Gruet *et al*., 2001**; **Awale *et al*., 2012**). *S. aureus* is recognized as the causative agent of variety of infections in animals and humans (**Mork *et al*., 2005**). In animals, the *S. aureus* continues to be one of the most important organisms related with clinical and subclinical bovine mastitis (**Moon *et al*., 1990**). Numerous types of bacteria have been also described as responsible agents of bovine mastitis (**Watts, 1988**; **Bradley, 2002**). For example, *Klebsiella* spp., *Pseudomonas aeruginosa*., *S. uberis*., *E. coli* and pyogenic bacteria are associated with clinical mastitis form while others microorganisms like *S. agalactiae*, CNS and *Enterococcus* spp are related with subclinical mastitis form (**Bradley, 2002**; **Barkema, 2009** ; **Awale *et al*., 2012**). The *S. aureus* remains of particular importance, because it is considered to be the most common etiological pathogen of contagious bovine mastitis (**Hiramatsu *et al.*, 2001; Barkema *et al.*, 2009**) as well as it's a primary reason for antibiotic use on farms (**Haran *et al*., 2012**) and it has the capacity to develop the resistance phenomenon to all antimicrobial agents (**Hiramatsu *et al.*, 2001; Barkema *et al.*, 2009**). In the developed and developing world alike, the resistance of infectious

microorganisms to therapeutics became a serious and a growing health threat because of the emergence of resistant organisms. In medicine and in veterinary medicine, antibiotics agent are used in order to fight against bacterial infections and must be chosen according to their effectiveness on the bacteria to be controlled, which can be tested by means of an antimicrobial susceptibility testing. The objective of this study was to evaluate in vitro the efficacy of a set of antimicrobials from different antibiotics family to the resistance phenomenon and detection of reliable resistance genes in *S. aureus* isolates originated from bovine mastitis, belonging to 37 herds in the northwest of Portugal.

Materials and methods

Samples

Bacterial strains: A series of 52 Staphylococcus isolates collected from 37 different dairy herds in the northwest of Portugal between the years of 2003 and 2008 and 2017 were included in this study. These isolates belonged to the microorganisms collection of the laboratory of Microbiology and Infectious Diseases, Department of Veterinary Clinics of the Institute of Biomedical Sciences Abel Salazar of the University of Porto, Porto, Portugal. Information about these isolates can be found in **Table 21**.

Table 21: Herds and year of collection of all 52 *S. aureus* isolates.

Herd	Isolates	Year
1	1 / 2 and 3	2003 / 2008
2	4	2003
3	5	2004
4	6	2003
5	7	2003
6	8	2003
7	9	2004
8	10 and 11	2003
9	12	2003
10	13	2004
11	14	2003
12	15	2003
13	16	2003
14	17	2004
15	18	2003
16	19	2004
17	20	2007
18	21	2008
19	22	2008
20	23 and 24	2008
21	25	2008
22	26	2008
23	27, 28 and 29	2017
24	30 and 31	2017
25	32 and 33	2017
26	34	2017
27	35	2017
28	36 and 37	2017
29	38, 39 and 40	2017
30	41	2017
31	42, 43, 44 and 45	2017
32	46	2017
33	47	2017
34	48	2017
35	49	2017
36	50	2017
37	51 and 52	2017

Antimicrobial susceptibility testing

The antimicrobial susceptibility test was conducted using the disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines (**CLSI, 2007**; **CLSI, 2014**). Bacteria were grown at 37°C for 18h to 24h on Columbia ANC agar supplemented with sheep blood 5% (bioMérieux, Marcy l'Etoile, France). Bacterial Colonies were re-suspended in 1 mL of 0.85% (w/v) sodium chloride (Merck Laboratories, Darmstadt, Germany) and adjusted to 0.5 McFarland in comparison with a McFarland standard (bioMérieux, Marcy l'Etoile, France). After that, Mueller–Hinton agar (Merck Laboratories, Darmstadt, Germany) plates were inoculated with the inoculum by dipping sterile cotton swabs into the bacterial suspension. After, the following antibacterial agents (all from bioMérieux, Marcy l'Etoile, France and from Oxoid, Thermo Fisher Scientific Inc., Lenexa, KS, U.S.A) were tested: gentamicin (10μg), tobramycin (10μg), amikacin (30μg) and kanamycin (30μg), all from aminosides family; tetracycline (TE; 30μg) (Tetracycline family); erythromycin (E; 15μg) and clindamycin (DA; 2μg) (Macrolides family); vancomycin (VA, 30μg) (Glycopeptides family) and other antibiotics class such as trimethoprim/sulfamethoxazole (1,25μg +23,75μg) (lipopeptides family). The inoculated agar plates with discs were incubated at 37°C for 24 hr. Following the incubation, the diameters of the inhibition zones were measured in millimetres and compared with the ranges suggested by the CLSI guidelines. To finish, the isolates were classified as susceptible, resistant, or intermediate on the basis of the size of the inhibition zone.

DNA extraction

Two colonies of each staphylococcal isolate previously streaked onto Columbia ANC agar supplemented with 5% of sheep blood (bioMérieux, Marcy l'Etoile, France) were inoculated in tubes with 10 mL of BHI and incubated at 37ºC for 24 h. After, tubes were centrifuged at 10000 g for 10 min and genomic DNA was extracted from pellets using the QIAamp® DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer instructions.

PCRs of *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *spc*, *tetM* , *tetK*, *ermT*, *vanA*, *lnuC*, *salA*, *dfrk* and *vgaC* resistance genes.

PCRs of *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *tetM* and *tetK* genes were performed as previously described (**Voss *et al.,* 1995**; **Strommenger *et al.,* 2003**; **Choi *et al.,* 2003**; **Luthje & Schwarz, 2007**; **Saha *et al.,* 2008**; **Feßler *et al.,* 2010**; **Kadlec & Schwarz, 2010**; **Chakraborty *et al.,* 2011**; **Nizami *et al.,* 2012**; **Gómez-Sanz *et al.,* 2013**; **Li *et al.,* 2013;**

Mahmood & Flayyih, 2014) using specific primers for the detection of the resistance genes (Table 10): *aac(6')aph(2'')* (aac_fw, 5′_GAAGTACGCAGAAGAGA-3′; aac_rev, 5′_ACATGGCAAGCTCTAGGA-3′; amplicon size: 491bp); *aph(3')-III* (aph_fw, 5′_AAATACCGCTGCGTA-3′; aph_rev, 5′_CATACTCTTCCGAGCAA-3′; amplicon size: 242bp); *ant(4')-Ia3* (ant_fw, 5′_AATCGGTAGAAGCCCAA-3′; ant_rev, 5′_GCACCTGCCATTGCTA-3′; amplicon size: 135bp); *tetK* (tetK_fw, 5′_GTAGCGACAATAGGTAATAGT-3′; tetK_rev, 5′_GTAGTGACAATAAACCTCCTA-3′; amplicon size: 360bp); *tetM* (tetM_fw, 5′_AGTGGAGCGATTACAGAA-3′; tetM_rev, 5′_CATATGTCCTGGCGTGTCTA-3′; amplicon size: 158bp); *spc* (spc_fw, 5′_ACCAAATCAAGCGATTCAAA-3′; spc_rv, 5′-GTCACTGTTTGCCACATTCG-3′; amplicon size: 561bp); *ermT* (ermT_fw, 5′ ATTGGTTCAGGGAAAGGTCA-3′; ermT_rev, 5′-GCTTGATAAAATTGGTTTTTGGA-3′; amplicon size: 536 bp); *vanA* (vanA_fw, 5′_ATGAATAGAATAAAAGTTGC-3′; vanA_rev, 5′_ TCACCCCTTTAACGCTAATA-3′; amplicon size: 1032bp); *SalA* (salA_fw, 5′_CGATGAACCAACAAACCACA-3′; salA rev, 5′_AGGACCGAACCTTGAAATGA-3′; amplicon size: 931bp); *lnuC* (lnuC_fw, 5′_ ATGAATAGAATAAAAGTTGC-3′; lnuC_rev, 5′ _TCACCCCTTTAACGCTAATA-3′; amplicon siz: 1100bp); *dfrK* (dfrK_fw, 5′ _GCTGCGATGGATAATGAACAG-3′; dfrK_rev, 5′_GGACGATTTCACAACCATTAAAGC-3′; amplicon size: 214bp); and *vga (C)* (vgaC_fw, 5′CCGTATGCCCAGAGTGAGAT-3′; vgaC_rev, 5′_TGCTTGGGAACAAGTCCTTC-3′; amplicon size: 671bp). Briefly, after DNA extraction all isolates were tested for *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *spc*, *tetM*, *tetK*, *ermT*, *vanA*, *lnuC*, *salA*, *dfrK* and *vgaC* using the set of primers as previously described, by PCR in a thermocycler C 1,000 (Bio-Rad, California, USA). The amplified products (*aac(6')/aph(2")*, -491 bp; *aph(3')-IIIa*, *ant(4')-Ia*, -242 bp; *ant(4')-Ia3*, -135 bp; *spc*, -561bp; *tetM*, -158bp; *tetK*, -360bp; *ermT*, -536bp; *vanA*, -1032bp; *dfrK*, -214bp; *vgaC*, -671bp and *lnuC*, 1100bp) (Nippon Genetics Europe GmbH, Duren, Germany) were analyzed on a 1.5% (w/v) agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Duren, Germany) and visualized under UV light (Bio-Rad, California, USA).

Results

Antimicrobial susceptibility

Overall, the level observed of antimicrobial resistance of the investigated strains in this study was high (**Table 22**). So, the results obtained of antibiotic susceptibility testing demonstrated that the resistance to tetracycline (Tetracycline family) was noted in the 39/52 of isolates (75.0%) while the resistance to erythromycin (Macrolides family) was showed in the 35/52 of isolates (67.3%) followed by a prevalence of 50.0% (26/52), 42.3% (22/52), 36.5% (19/52), 32.7% (17/52), 28.8% (15/52), 23.1% (12/52), 21.2% (11/52) of resistance to vancomycin, amikacin, kanamycin, clindamycin, gentamycin, tobramycin and trimetoprin/sulfamethoxazole, respectively (**Table 22**). Furthermore, intermediate resistance against vancomycin (34.6%, n=18), trimetoprin/sulfamethoxazole (28.8%, n=15), tobramycin (26.9%, n=14), gentamycin (21.2%, n=11), tetracycline (13.5%, n=07), erythromycine (13.5%, n=07), kanamycin (13.5%, n=07), clindamycin (7.7%, n=04) and amikacin (7.7%, n=04) was observed (**Table 22**).

Table 22: Antimicrobial tests performed to all *S. aureus* isolates from bovine mastitis against antibiotics by the disk diffusion method following CLSI guidelines.

No. of Isolates	Antimicrobial agent (s)								
	Tetracycline	Erythromycin	Clindamycin	Vancomycin	**Trimethoprim/ Sulfamethoxazole**	**Gentamicin**	**Tobramycin**	**Kanamycin**	**Amikacin**
1	R	R	S	S	S	S	S	S	S
2	R	R	S	S	S	S	S	S	S
3	R	R	S	S	S	S	S	S	S
4	R	R	S	S	S	S	S	S	S
5	R	R	S	S	S	S	S	S	S
6	R	R	S	S	S	S	S	S	S
7	R	R	S	S	S	S	S	S	S
8	R	R	S	S	S	S	S	S	S
9	R	R	S	S	S	S	S	S	S
10	S	S	S	S	S	S	S	S	S
11	R	R	S	S	S	S	S	S	S
12	R	R	S	S	S	S	S	S	S
13	R	R	S	S	S	S	S	S	S
14	R	R	S	S	S	S	S	S	S
15	R	R	S	S	S	S	S	S	S
16	R	R	S	S	S	S	S	S	S
17	R	R	S	S	S	S	S	S	S
18	R	S	S	S	S	S	S	S	S
19	S	S	S	S	S	S	S	S	S
20	R	R	S	S	S	S	S	S	S
21	S	R	S	S	S	S	S	S	S
22	S	S	S	S	S	S	S	S	S
23	S	S	S	S	S	S	S	S	S
24	R	R	S	S	S	S	S	S	S
25	R	R	S	S	S	S	S	S	S
26	S	S	S	S	S	S	S	S	S
27	I	R	R	R	I	R	R	R	R
28	R	R	I	I	R	R	I	I	R
29	R	R	R	I	R	I	R	R	R
30	R	R	R	I	I	I	I	R	R
31	I	R	R	I	I	I	I	R	R
32	R	R	R	I	R	R	I	R	R
33	R	R	R	I	R	R	R	R	R
34	R	R	R	I	I	R	R	R	R
35	R	R	R	R	R	R	R	R	R
36	R	I	I	I	I	R	I	R	R
37	R	R	I	I	I	I	I	I	R
38	R	I	R	I	R	R	R	R	R
39	R	R	R	I	R	R	R	R	R
40	R	R	R	I	R	I	R	I	R
41	I	I	R	I	R	I	I	I	I
42	R	R	R	I	I	R	R	R	R
43	R	R	R	R	I	R	R	R	R
44	I	I	S	I	I	I	I	R	I
45	I	S	S	R	I	I	I	I	I
46	I	S	S	R	R	I	I	I	I
47	R	I	S	R	I	I	I	I	R
48	I	I	S	R	I	R	I	R	R
49	R	S	R	I	I	R	R	R	R
50	R	S	R	R	R	R	R	R	R
51	R	R	R	I	I	R	I	R	R
52	R	I	I	I	I	I	I	R	R
Susceptible Isolates									
No.	06/52	10/52	31/52	8/52	26/52	26/52	26/52	26/52	26/52
%	**~11.5**	**~19.2**	**~59.6**	**~15.4**	**~50.0**	**~50.0**	**~50.0**	**~50.0**	**~50.0**
IntermediateIsolates									
No.	7/52	7/52	4/52	18/52	15/52	11/52	14/52	7/52	4/52
%	**~13.5**	**~13.5**	**~07.7**	**~34.6**	**~28.8**	**~21.2**	**~26.9**	**~13.5**	**~7.7**
Resistant Isolates									
No.	39/52	35/52	17/52	26/52	11/52	15/52	12/52	19/52	22/52
%	**~75.0**	**~67.3**	**~32.7**	**~50.0**	**~21.2**	**~28.8**	**~23.1**	**~36.5**	**~42.3**

Antimicrobial resistance genes profile

The antimicrobial resistance genes of 33 (63.5%), 30 (57.7%), 16 (30.8%), 10 (19.2%), 8 (15.4%), 3 (5.8%) and 1 (1.9%) strains out of 52 isolates studied were detected positive using PCR by amplification of *ant(4)-I-a, tetM*, *aph(3')-III-a*, *dfrK*, *tetK*, *lnuC* and *spc* genes, respectively (**Table 23, Figures 46, 47, 48** and **49**). Moreover, the remaining tested genes such as *aac(6)/aph(3)*, *tetK*, *vanA*, *salA*, *vgaC* and *ermT*, were negative (**Table 23**).

Table 23: Antimicrobial resistance genes profile.

Genes Type / No. Isolates	*Dfrk*	*Tet*M	*Tet*K	*aph (3')-III*	*Ant (4')–Ia3*	*aac (6')-aph(2')*	*erm*T	*Lnu*C	*Sal*A	*Vga*C	*Spc*	*van*A
1	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
2	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
3	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Positive	Negative
4	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
6	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
8	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
9	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
10	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
11	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
12	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
13	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
14	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
15	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
16	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
17	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
18	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
19	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
20	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
21	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
22	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
23	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
24	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
25	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
26	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
27	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
28	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
29	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
30	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative
31	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative
32	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
33	Negative	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
34	Negative	Positive	Positive	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative
35	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
36	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
37	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
38	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
39	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
40	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
41	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
42	Positive	Negative	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
43	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
44	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
45	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
46	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
47	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
48	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
49	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
50	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
51	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
52	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
% Positive Isolates	**19.2**	**57.7**	**15.4**	**30.8**	**63.5**	**00.0**	**00.0**	**5.8**	**00.0**	**00.0**	**01.9**	**00.0**
% Negative Isolates	**80.8**	**42.3**	**84.6**	**68.2**	**36.5**	**100.0**	**100.0**	**99.4**	**100.0**	**100.0**	**100.0**	**100.0**

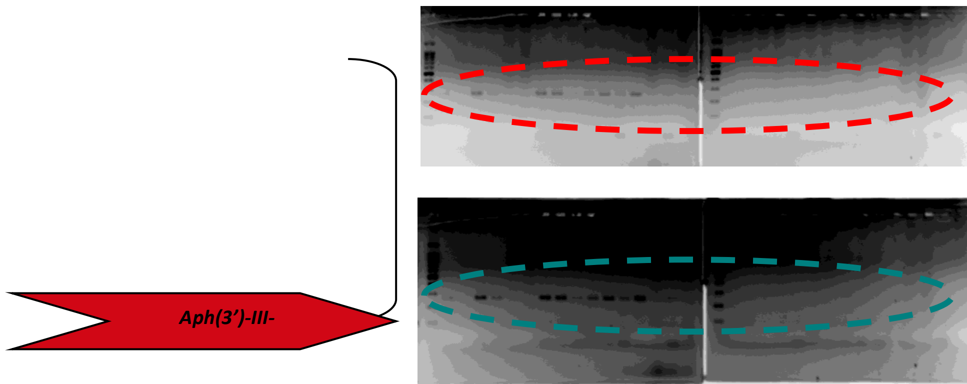

Figure 46: Amplification of *Aph(3')-III-a* gene by using PCR method.

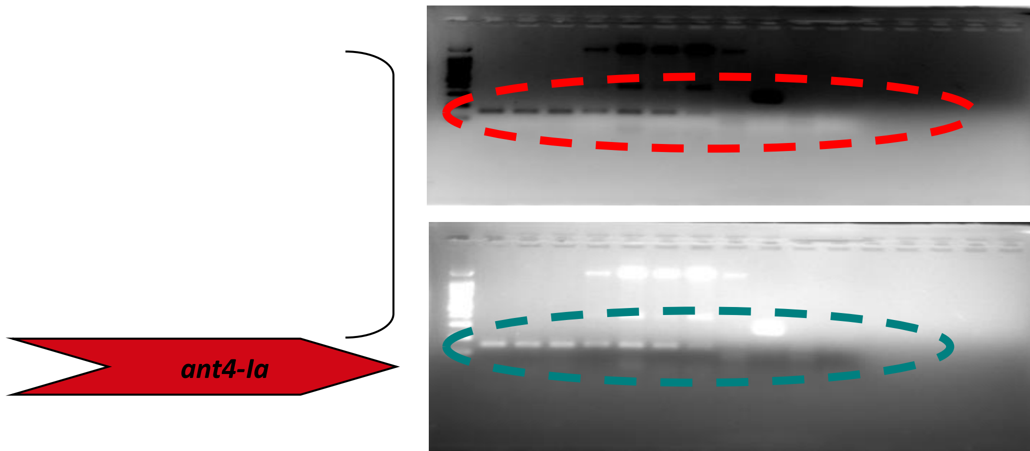

Figure 47: Amplification of *Ant4-Ia* gene by using PCR method.

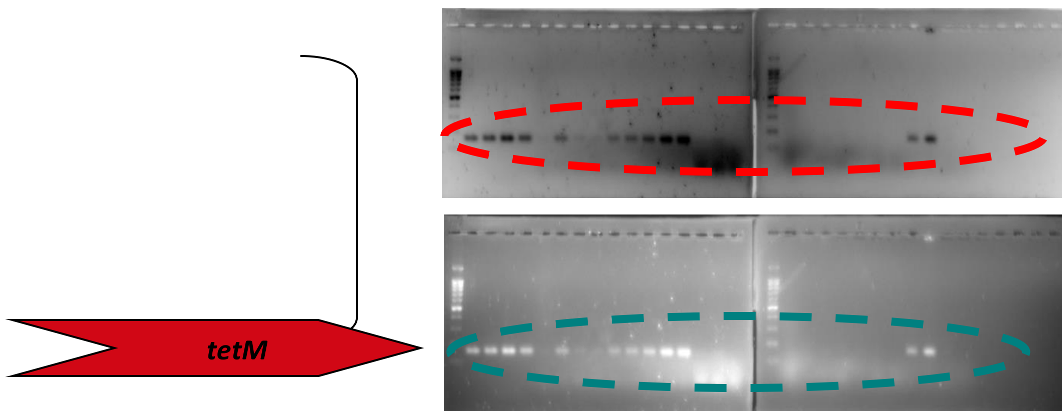

Figure 48: Amplification of *tetM* gene by using PCR method.

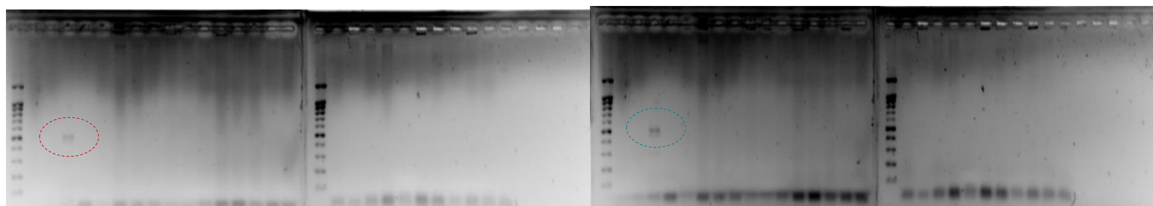

Figure 49: Amplification of *spc* gene by using PCR method.

The no correspondence between genotypic and phenotypic findings has been also observed, especially for aminoglycoside modifying enzymes and for *tet* and *ermT* genes (**Table 24**).

Table 24: Correspondence level between phenotypic and genotypic findings.

Antibiotics and genes / No. Isolates	Tested antibiotics	Tet genes detected		Tested antibiotics from aminoglycosides				Amino-modifying enzymes genes detected		
	Tetracycline	*tet* M	*tet* K	Gentamicin	Tobramycin	Kanamycin	Amikacin	*aph (3') – III*	*ant (4') – Ia3*	*Aac (6')-aph (2')*
1'	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	**Positive'**	Negative
2'	R	Positive	Negative	S	S	S	S	Negative	**Positive'**	Negative
3'	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	**Positive'**	Negative
4'	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	**Positive'**	Negative
5'	R	Negative	Negative	S	S	S	S	Negative	**Positive'**	Negative
6'	R	Negative	Negative	S	S	S	S	Negative	**Positive'**	Negative
7'	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	**Positive'**	Negative
8	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
9	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
10	S	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
11	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
12	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
13	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
14	R	Positive	Negative	S	S	S	S	Negative	Negative	Negative
15	R	Negative	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
16	R	Negative	Negative	S	S	S	S	Negative	Negative	Negative
17	R	Negative	Negative	S	S	S	S	Negative	Negative	Negative
18	R	Negative	Negative	S	S	S	S	Negative	Negative	Negative
19	S	Negative	Negative	S	S	S	S	Negative	Negative	Negative
20	R	Negative	Negative	S	S	S	S	Negative	Negative	Negative
21	S	Negative	Negative	S	S	S	S	Negative	Negative	Negative
22	S	Negative	Negative	S	S	S	S	Negative	Negative	Negative
23	S	Negative	Negative	S	S	S	S	Negative	Negative	Negative
24	R	Positive	Negative	**S**	**S**	**S**	**S**	**Positive**	Negative	Negative
25	R	Positive	Negative	S	S	S	S	Negative	Negative	Negative
26	S	Negative	Negative	S	S	S	S	Negative	Negative	Negative
27	I	Positive	Negative	R	R	R	R	Negative	Positive	Negative
28	R	Positive	Negative	R	I	I	R	Positive	Positive	Negative
29	R	Negative	Positive	I	R	R	R	Negative	Positive	Negative
30	R	Positive	Negative	I	I	R	R	Negative	Positive	Negative
31	I	Positive	Negative	I	I	R	R	Negative	Positive	Negative
32	R	Positive	Negative	R	I	R	R	Negative	Positive	Negative
33	R	Positive	Positive	R	R	R	R	Negative	Positive	Negative
34	R	Positive	Positive	R	R	R	R	Negative	Positive	Negative
35	R	Positive	Positive	R	R	R	R	Negative	Positive	Negative
36	R	Positive	Positive	R	I	R	R	Positive	Positive	Negative
37	R	Negative	Positive	I	I	I	R	Negative	Positive	Negative
38	R	Positive	Positive	R	R	R	R	Negative	Positive	Negative
39	R	Positive	Negative	R	R	R	R	Positive	Positive	Negative
40	R	Negative	Negative	I	R	I	R	Negative	Positive	Negative
41	I	Negative	Negative	I	I	I	I	Negative	Positive	Negative
42	R	Negative	Negative	R	R	R	R	Positive	Positive	Negative
43	R	Positive	Negative	R	R	R	R	Negative	Positive	Negative
44	I	Positive	Negative	I	I	R	I	Negative	Positive	Negative
45	I	Positive	Negative	I	I	I	I	Negative	Positive	Negative
46	I	Negative	Negative	I	I	I	I	Negative	Positive	Negative
47	R	Positive	Negative	I	I	I	R	Negative	Positive	Negative
48	I	Negative	Negative	R	I	R	R	Negative	Positive	Negative
49	R	Negative	Positive	R	R	R	R	Negative	Positive	Negative
50	R	Positive	Negative	R	R	R	R	Negative	Positive	Negative
51	R	Negative	Negative	R	I	R	R	Negative	Positive	Negative
52	R	Negative	Negative	I	I	R	R	Negative	Positive	Negative
% of positive detected genes/% of Resistant or Intermediate Isolates										
Pourcentage of correspandance	-	**63.0%** (29/46)	**17.4%** (8/46)	-	-	-	-	**7.0%** (4/26)	**100.0%** (26/26)	**0.0%** (0/26)

Discussion

The improvement of antibiotics in order to treat infections caused by bacteria has generally conducted to improve health (**Voss & Doebbeling, 1995**) but the use of these antibiotics in animals and also in humans is the primary cause of the appearance of resistance phenomenon to antimicrobials in bacteria, which is a public health hazard (**Levy & Marshall, 2004**). The antibiotics resistance caused by *S. aureus* is widely spreading because of extensive use of these antimicrobial agents in bovine mastitis (**Barkema *et al.*, 2006** ; **Gao *et al.*, 2012**).

The present study leaded to evaluate the efficacy of a set of antimicrobials presented from different of antibiotics family to the resistance phenomenon caused by *S. aureus* originated from bovine mastitis, case of some herds in the northwest of Portugal. The presented findings indicated a considerable prevalence of antimicrobial resistant strains among *S. aureus* isolated from bovine mastitis disease in the northwest of Portugal. Similar or different to studies conducted by other authors from another region of the world, the highest resistance prevalence was found for tetracycline (75.0%) followed by erythromycin (67.0%), vancomycin (50.0%), amikacin (42.3%), kanamycin (36.5%), clindamycin (32.7%), gentamycin (28.8%), tobramycin (23.1%) and trimethoprim/sulfamethoxazole (21.2%) (**Table 22**). Furthermore, intermediate resistance against vancomycin (34.6%, n=18), trimetoprin/sulfamethoxazole (28.8%, n=15), tobramycin (26.9%, n=14), gentamycin (21.2%, n=11), tetracycline (13.5%, n=07), erythromycine (13.5%, n=07), kanamycin (13.5%, n=07), clindamycin (7.7%, n=04) and amikacin (7.7%, n=04) was observed (**Table 22**). The antimicrobial resistance genes of 63.5%, 57.7%, 30.8%, 19.2%, 15.4%, 5.8% and 1.9% of strains studied were detected positive for *ant(4)-I-a, tetM*, *aph(3')-III-a*, *dfrK*, *tetK*, *lnuC* and *spc* genes, respectively. Moreover, the remaining tested genes such as *aac(6)/aph(3)*, *tetK*, *vanA*, *salA*, *vgaC* and *ermT*, were detected negative (**Table 23**). In Portugal, the published studies on bovine mastitis caused by *S. aureus* are few. In this regards, the present study showed often a similar or different results for some antibiotics to that reported in other studies especially in the northwestern, central and southern regions and in the central region of Portugal (**Nunes *et al.*, 2007**; **Rocha *et al.*, 2014**). On dairy farms, antimicrobials drugs such as gentamicin (**Burrows *et al.*, 1987**), erythromycin, tetracycline, penicillin, streptomycin and cephalosporin, among others, are used in order to treat and prevent mastitis affecting dairy cows that is caused by variety of Gram-negative and Gram-positive bacteria (**Oliver & Murinda, 2012**). The resistance observed to erythromycin in this study must be of concern because macrolide antibiotics were potentially best for the treatment of Gram-positive bacteria mastitis (**Preeza, 2000**). However, an antimicrobial resistance of

54.8%, 52.1%, 79.9%, 74.4%, 77.2% and 91.8% was described in Chinese dairy farms for gentamicin, chloramphenicol, tetracycline (oxytetracycline), erythromycin, clindamycin and trimethoprim/sulfamethoxazole, respectively (**Wang *et al*., 2015**). These findings stay higher than what we observed here (28.8% for gentamicin, 32.7% for clindamycin, 75.0% for tetracycline, and 67.0% for erythromycin). The variation observed in resistance prevalence of *S. aureus* mastitis in Chinese and Portuguese dairy farms in the present investigation could be due to differences in practice conditions, as well as a lack of a mastitis-control program in each country. The use of erythromycin antibiotic is approved in veterinary practice in China for more than half a century for the treatment of Staphylococcal mastitis but the erythromycin-resistant *S. aureus* isolates have become an increasingly recognized problem in many parts of China in the last two decades according to **Wang *et al*., (2008)**. When comparing our results with those reported by **Ranjan *et al*., (2010)**, we found a high prevalence rate of resistance to kanamycin (43.9%) and a low rate for gentamicin (22.7%), and for amikacin (12.8%). These differences could be associated to the microorganisms selection because the various isolates adopted in this study were identified as *S. aureus*, coagulase negative *Staphylococcus* spp., *E. coli*, *Pseudomonas* spp., *Streptococci* spp., *Klebsiella* spp, *Bacillus* spp., Yeast while our study was focused only on *S. aureus*. At the same time, our findings stay also similar (for tetracycline (76.7%) and clindamycin (34.9%)) or different that what has already reported by **Jamali *et al*., (1997)** in Iranian herds (for erythromycin (39.5%), chloramphenicol, trimethoprim-sulfamethoxazole (11.6% each), gentamicin (7.0%), streptomycin (2.3%), and for kanamycin and tobramycin (0.0% each)). The detection of *S. aureus* resistance for multi antimicrobials within Iranian herds could be less frequent in comparison to Portuguese herds. **Sakwinska *et al*., (2011)** showed that the frequency resistance in Swiss isolates are 3.1%, 1.0%, 1.0%, 0.5% and 0.0% for tetracycline, gentamicin, kanamycin, erythromycin and vancomycin, respectively. Moreover, in French isolates, the frequency resistance is 5.3%, 2.7%, 0.0%, 0.0%, 0.0% for tetracycline, gentamicin, kanamycin, erythromycin and vancomycin; respectively. These findings remain lower than our results obtained especially for these antimicrobial agents above described (75.0%, 28.8%, 36.5%, 67.0%, 50.0%), respectively. Moreover, in Burdur province of Turkey, **Turutoglu *et al*., (2006)**, showed high resistance prevalence than those previously described in our study especially for gentamicin (56.3% n=58) and trimethoprim/sulfa-methoxazole (45.6% n=47); while a similar prevalence for tetracycline (75.0%) was noted in case of oxytetracycline (76.7% n= 63). This could be attributed to misuse of antibiotics agents noted in Turkey, where these are practically dispensed without a prescription (**Turutoglu *et al*., 2006**) in contrast to Portugal where the antibiotics are in general dispensed with a prescription.

In addition, the incomplete or wrong treatment of animals in cases of mastitis also contributes significantly to the development of bacterial resistance against antimicrobial used for treatment according to **Turutoglu *et al.*, (2006)** whereas some other authors are suggested that exposure to antimicrobials for a long-term (for example, continuous use of dry cow treatment) considerably increases the resistance level to antimicrobial (**Pol & Ruegg, 2007**; **Daniele *et al.*, 2014**). In comparison, among the mastitis in cattle and buffaloes isolates examined by **Sharma *et al.*, (2015)** in Mathura in India, the prevalence of resistance found only to vancomycin was 88.8%, followed by streptomycin (44.4%) and 22.2% for clindamycin, gentamicin and tetracycline. As far as sensitivity is concerned, **Sharma *et al.*, (2015)** showed 100.0% sensitivity to amikacin and high sensitivity (88.8%) towards chloramphenicol and erythromycin. In Eastern Poland, **Swzeda *et al.*, (2014)**, showed different and less rates of tetracycline resistance (1.6%) and also for erythromycin resistance (2.4%) in comparison to those found in our study, achieving also, to a little resistance prevalence (1.6%) for clindamycin and (0.8%) for trimethoprim/sulfamethoxazole than we found (32.7% for clindamycin and 21.2% for trimethoprim/sulfamethoxazole). Furthermore, in a study conducted with human and bovine *S. aureus* strains, gentamicin, tetracycline, erythromycin, clindamycin, trimethoprim/sulfa-methoxazole and vancomycin conferred an antimicrobial resistance of 61.4%, 53.5%, 32.4%, 33.3%, 9.6% and 7.9% against human strains, respectively; while an antimicrobial resistance of 20.0%, 6.2%, 9.3%, 7.0%, 0.0%, 0.0% was found respectively for the same antimicrobial agents against bovine strains. The findings we obtained for clindamycin (32.7%) are in accordance to that previously described by **Tel *et al.*, (2012)** in this study, but just for *S. aureus* originated from human strains. It appears from these previous studies described that the reported resistances levels vary significantly between countries that have shown a variable incidence of inducible resistance among the tested *S. aureus* isolated from bovine mastitis or from human. Moreover, the rates of resistance to tetracycline (75.0%), erythromycin (67.3%), vancomycin (50.0%), amikacin (42.3%), kanamycin (36.5%), clindamycin (32.7%), gentamycin (28.8%), tobramycin (23.1%) and trimetoprin/sulfamethoxazole (21.2%) were less than those reported in other reports especially in case of human isolates. For example, **Rahimi *et al.*, (2016)** showed a rates of resistance arriving to 99.0% for kanamycin and tobramycin, 97.0% for erythromycin, 90.0% for clindamycin, for 82.0% amikacin, around 66.0% for trimetoprin/sulfamethoxazole and 47.8% for gentamycin. This difference could be explained by the high level of these antibiotics being prescribed for the treatment of human more than animal infections. Furthermore, this indicates that these antimicrobials agents are no longer effective antibiotics against *S. aureus* human infections.

The results obtained from the PCR screening for antimicrobial resistance genes reflected in general that a prevalence of 63.5%, 57.7%, 30.8%, 19.2%, 15.4%, 5.8% and 1.9% was detected positive using PCR by amplification of *ant(4)-I-a, tetM*, *aph(3')-III-a*, *dfrK*, *tetK*, *lnuC* and *spc* genes, respectively. Moreover, the remaining tested genes such as *aac(6)/aph(3)*, *tetK*, *vanA*, *salA*, *vgaC* and *ermT*, were negative for all tested isolates. Aminoglycosides are known to be one of the classes of antibiotics that play a significant role in the treatment of staphylococcal infections (**Hauschild *et al.*, 2008**). Concerning PCRs results for aminoglycoside-modifying enzymes (AMEs), we detected that *ant(4)-I-a* was found in 63.5% of isolates whereas *aph(3')-III-a* (with 57.7%) was also among the most prevalent genes of AMEs as previously described by **Turutoglu *et al.*, (2009**); and any isolates was not found positive for *aac(6')/aph(2")*. According authors, the major resistance mechanism to aminoglycosides family is generally conferred by an inactivation of antimicrobials by AMEs that are decoded by genetic elements. The *aac (6')-Ie + aph (2'')*, ant (4')-Ia, *aph (3')-IIIa*, and *ant (6)-Ia* genes that encode aminoglycoside-6'-N-acetyltransferase/2"-O-phosphoryltransferase, aminoglycoside-4'-O nucleotidyltransferase I, aminoglycoside-3'-O- phosphoryltransferase III, and streptomycin modifying enzyme, respectively, are therefore the mainly considerable genes in this context (**Hauschild *et al.,* 2008; Turutoglu *et al.,* 2009; Schwitz, 1999; Vakulenko & Mobashery, 2003; Rahimi *et al.*, 2016**). In general, our findings obtained for AME are different to those reported by other authors who showed that *aac(6')-aph(2")* gene was much higher in comparison to that of the other two AME genes, *aph(3')-IIIa* and *ant(4')-Ia* in *S. aureus* isolated from animals (**Goni *et al.,* 2004; Schnellmann *et al.*, 2006**). In the literature, the resistance to gentamicin and kanamycin is conferred via the gene *aacA-aphD* [*aac(6')-Ie – aph(2')-Ia*]. In addition, the *ant(4')-Ia* gene have been described to confer resistance to kanamycin and tobramycin while streptomycin resistance is conferred by *ant(6)-Ia* gene (**Jamali *et al.*, 1997**). As previously reported, the concomitant resistance showed to tobramycin and kanamycin and this associated to gentamicin are mainly mediated by a bifunctional enzyme displaying APH(2'') and AAC(6') activity (**Ubukata *et al.*, 1984; Matsumara *et al.*, 1984**). The *aac(6')-Ie aph(2'')* gene encodes this bifunctional enzyme and is determined (encoded) on composite transposon Tn*4001*. Moreover, the resistance showed to neomycin, kanamycin, tobramycin and amikacin is generally conferred by an ANT(4')-I enzyme encoded by *ant(4')-Ia* by that inactivates these antibiotics (**Paulsen *et al.*, 1997; Schwitz *et al.*, 1999; Vakulenko & Mobashery, 2003; Jones *et al.*, 2006; Shahsavan *et al.*, 2012; Rahimi, 2016**) while the APH (3')-III, enzyme inactivates neomycin (**Jones *et al.*, 2006; Shahsavan *et al.*, 2012; Rahimi, 2016**).

In many parts of the world, tetracycline antibiotics still remain as one of the first-line treatment accorded for a number of infections (**Roberts, 2003; Jones *et al.*, 2006; Shahsavan *et al.*, 2012**). In the literature review, we note a limited data amount regarding the prevalence rate of *tet* genes among *S. aureus* strains isolated from bovine mastitis especially in north of Portugal. The tetracycline antibiotic family tested in this study and the found antimicrobial resistance especially for tetracycline against *S. aureus* isolates suggests a direct link to a variety different *tet* genes (**Schmitz *et al.*, 2001**), including *tetK* and *tetM* according to **Warsa *et al.*, (1996)** and *tetL* and *tetO* according to **Trzcinski *et al.*, (2000)**. Our results show that the *tetM* was the most prevalent genes found in our isolates (30/52) in contrast to *tetK* gene that was only detected in (08/52) of the isolates tested.

In this regards, we can conclude that most common tetracyclines resistance mechanism presented, in the current study, could be also conferred by *tetM* and *tetK* gene, respectively; as previously described among tetracycline resistant MRSA from Malaysia, Turkey, and most European countries, in which this resistance mechanism by these genes was predominant, but in contrast to North America where there is a high frequency rate of the *tetK* gene among MRSA isolates (**Sekiguchi *et al.*, 2004; Ardic *et al.*, 2005; Jones *et al.*, 2006; Lim *et al.*, 2012; Lozano *et al.*, 2013**).

In general, these findings could be explained by the two mechanisms of tetracycline-resistance that have been identified in Staphylococcus species: the first is associated with active efflux resulting from the acquisition of the *tetK* and *tetL* genes located on a plasmid while the second is related to ribosomal protection mediated by *tetM* or *tetO* determinants located on either a transposon or the chromosome (**Bismuth *et al.*, 1990; Warsa *et al.*, 1996; Schwarz *et al.*, 1998**). It is confirmed also that *tetK* specifies resistance to tetracycline but not to minocycline whereas *tetM* specifies resistance to both antibiotics (**Warsa *et al.*, 1996**). According to **Levy (2002)**, the *tetM* gene, produces, for example, a protein that can inhibit binding of tetracycline. This phenomenon is much frequently encountered in case of methicillin resistant *Staphylococcus aureus* (MRSA), which are responsible of the penicillin binding protein PBP2 production encoded by *mecA* gene (**Hartman & Thomasz, 1984; Paul *et al.*, 2002**). In general, antibiotic of tetracycline and also most of its derivatives have been shown to bind to ribosomes and selectively inhibit protein synthesis, a few of its derivatives that cannot act with this way (**Speer *et al.*, 1992**). Coexistence of *tetM* and *tetK* genes among the *S. aureus* isolates was detected in this study, as well as in the study of **Trzcinski *et al.*, (2000)**. Our results are also consistent with those reporting the *tetL* and the *tetO* genes to be rarely detected in *S. aureus* isolates.

Our result concerning the detection of resistance gene *vanA* to vancomycin was negative by using PCR test in all isolates that showed also a resistance of 50% to vancomycin antibiotic. This finding is in contrast to findings previously described for vancomycin antibiotic prevalence (**Sakwinska *et al.*, 2011; Szweda *et al.*, 2014**). This high rate resistance observed to vancomycin could be associated to other van genes as previously discussed by many authors (**Moubareck *et al.*, 2009; Perichon & Courvalin, 2009; Saadat *et al.*, 2014**). In general, the use of glycopeptides antibiotics was approved in order to treat the infections caused by gram-positive bacteria in case of resistance or allergy to other antimicrobial agents (**Depardieu *et al.*, 2004**).

<u>In this study, we investigated also whether the findings that were obtained from phenotypic and genotypic testing are in concordance or not?</u>

When comparing the resistance or intermediate phenotypes obtained and genotypes detected in this study, a correspondence of 63.0% (29/46 total resistant or intermediate isolate) and 17.4% (8/46) was observed between resistant or intermediate and PCR positive isolates findings for *tetM* and *tetK,* respectively. Moreover, in case of aminoglycosides antimicrobials tested, a correspondence of 100.0% (26/26) and 11.5% was noted for *ant(4')-Ia* and for *aph (3') – III genes*, respectively (**Table 24**).
This discrepancy between genotypic and phenotypic findings in these isolates could be as the result of the presence of other genes that could be responsible for the resistance associated to some antibiotics more than others in each antimicrobial family. Similarly, other researchers also showed that the presence of a certain resistance gene was not of necessity an indicator of antibiotic resistance (**Xu *et al.,* 2014**). A study by **Ma *et al.,* (2018)** showed that the inconsistency between phenotype and genotype happened in most *S. aureus* isolates tested, which could associate to other undetected resistance genes, or to the influence of other genetic factors, or to the environmental conditions. For example, the non correspondence that was observed for erythromycin in phenotypic testing (81.0%) against genotypic testing that was found negative in all isolates tested for *ermT* gene. This finding is probably due to other erm genes (*ermA*, *ermB*, *ermC*, *ermF*, *ermT* genes) (**Murphy, 1985; Chung *et al.*, 1999; Petra & Schwarz, 2007; Ding *et al.*, 2012**) and also to *msrA*, *msrB* and *mphC* genes **(Jamali *et al.*, 1997)** that are confirmed to be also responsible for erythromycin-resistance along with *ermT*.
In this context, **Ding *et al.*, (2012)** suggested that erythromycin-resistance was caused mainly by methylase encoded by *ermB* and *ermC* genes. At this point, other studies have previously

reported that the majority of this erythromycin-resistance from diverse cases of bovine mastitis could be related to different mechanisms like target region modification by methylation or mutation (**Chung *et al.*, 1999; Leclercq, 2002; Wang *et al.*, 2008**), active efflux mechanism (**Wang *et al.*, 2008**; **Ding *et al.*, 2012**), loss of permeability (**Leclercq, 2002; Dingwell *et al.*, 2003**; **Wang *et al.*, 2008**) and drug inactivation (**Fthenakis, 1998**; **Wang *et al.*, 2008**). Similar explanation could be attributed to findings obtained for tetracycline and aminoglycosides antibiotics and the tet and AMEs genes in phenotypic and genotypic testing.

Moreover, some isolates were found to be positive for some genes while there were susceptible to all antibiotics tested in this study for these genes such as case of isolate No.10 for *tetM* and isolates No. 1, 2, 3, 4, 6, 7 and isolates No. 1, 3, 4, 7, 8, 9, 10, 11, 12, 13, 15, 24 for *ant (4') – Ia3* and *aph (3') – III*, respectively. To our knowledge, there are not enough data explaining this phenomenon, but **Haveri *et al.*, (2005)** have suggested that phenotypically susceptible isolates that carry resistance genes should be considered as potentially resistant. Our suggestion to explain this phenomenon, could be for example, that these cows could have been administrated other antibiotics of the tetracyclines or of the aminoglycosides family in place of conventional antibiotics, or that the presence of a mutation or a set of mutations could have occurred at the level of the gene encoded for this resistance. Furthermore, besides detecting the gene, further studies on the evaluation of the gene expression/unexpression must be done to better explain this phenomenon.

In conclusion, all tested antibiotics in this study showed a high efficacy towards all the S. aureus strains collected during the period from 2003 to 2008. Whilst, all the strains collected in 2017 demonstrated almost all phenotypic resistance or intermediate against all antibiotics, showing an alarming and dramatic evolution of appearance of new resistant strains. An increasing phenotypic resistance of bovine mastitis *S. aureus* against tested antibiotics, with a high prevalence of phenotypic resistance (≥ 50.0%) among tetracycline, erythromycin and vancomycin was observed. Relatively to tetracycline, erythromycin and for some aminoglycosides (case of gentamicin, amikacin, kanamycin and tobramycin), macrolides (case of clindamycin) and for lipopetides (case of trimethoprim/sulfamethoxazole) obtained results, we suggest that this is an alarming situation and could create a serious challenge to the bovine mastitis therapy in Portugal. Moreover, the *tet(M)*, *aph(3')-IIIa* and *ant(4`)-Ia*, spc genes were the most prevalent antibiotic resistance genes detected.

These findings could be advantageous for design a new specific program for bovine mastitis disease control caused by *S. aureus* in the northwest region of Portugal. Furthermore, also suggests that we must change rapidly our habitual vision and reaction to the better antibiotic to

choose and apply for treatment before humanity declares a total capitulation to new infections caused by *S. aureus* in few coming years.

General Conclusion

In veterinary and human medicine, antimicrobial agents play an important role in the therapy of bacterial infections. But, resistance of infectious microorganisms to therapeutic agents remains a serious- and growing treat in human and animal health, notably in the developed and developing world alike. *S. aureus*, Gram positive bacteria considered to be an important human pathogen and known as one of the most important agent associated with bovine mastitis worldwide is commonly involved in the inflammation of the mammary gland, usually developed in response to an intramammary bacterial infection: bovine mastitis. This latter caused by staphylococcal or other agents, is recognized as an endemic disease and considered to be the most prevalent and expensive disease in the dairy farms and still remaining as an economically relevant problem to the dairy industry in several countries.

In the world, antimicrobial resistance of *S. aureus* is extensively spreading due to extensive utilization of antibacterial agents in bovine mastitis. This antimicrobial resistance phenomenon is developed by the pathogens, and this could represent one of main reasons of low cure rate of mastitis. The use of veterinary drugs by veterinarians are sometimes imperative and plays a major role in the control of diseases in cattle populations, good management and preventive practices in the herds can help the reduction of disease expression affecting this vital sector and consequently the need to resort to drugs that should be done wisely. Thus, in order to gather a set of information on Beta-lactam family concerning the resistance of *S. aureus*, originated from clinical and sub-clinical mastitis disease, against this prominent antimicrobial family; the first objective of our study in its first part of this thesis was to investigate the susceptibility of a set of antibiotics representing all groups of the β-lactam family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, during years 2003-2004, 2007-2008 and 2017. We aimed also to evaluate the detection of the presence of *blaZ* and *mecA* resistance genes in all strains by using specific PCR methods as well as the phylogenetic analysis of the *blaZ* gene in the selected isolates.

The obtained results show that the 26 strains collected from 2003 to 2008, demonstrate a resistance to aztreonam (Monobactames group) in all of isolates (n=26; 100.0%), whereas the resistance to penicillin (Penicillins G group) was found considerably higher reaching 76.9% (n=20) followed by resistance to ampecillin (Aminopenicillins group) with a level of 73.0% (n=19). The susceptibility test performed for other antibiotics within this group showed that all rest of strains was sensitive 100.0% (n=26) to amoxicillin associated to gluvalunic acid while the same one strain resistant to oxacillin was also found resistant to amoxicillin (Penicillins M group) presenting 3.8% (n=1). Unlike, all strains showed a significantly higher susceptibility

100.0% (n=26) for other antimicrobial groups studied in parallel as cefazolin (Cephalosporines group); piperacillin (Ureidopenicillins group); imipenem (Carbapenems group) and ticarcillin (Carboxypenicillins group). However, the PCR results obtained of all 26 *S. aureus* strains carrying *bla*Z gene that was performed through successful amplification of (377bp) specific products, show that all these strains are negative for *mecA* genes, except for isolate 25 that was the only one positive for *mecA*. In addition, the phylogenetic analysis of the detected *bla*Z placed the isolates, collected during 2003-2004- and 2007-2008 periods, in 3 different clusters that are closely related to other different bovine mastitis and human *S. aureus* strains. We followed also the evolution of resistance to antibiotic groups within β-lactam family, such as Penicillin G (penicillin (10 U), Penicillin M (oxacillin (1 µg), Aminopenicillins (ampicillin (10 µg), amoxicillin associated to clavulanic acid (20 µg + 10 µg), Ureidopenicillin (piperacillin (100 µg) and Cephalosporin (cefazolin (30 µg) in northwest of Portugal. For this, we assayed a new strains collection from cows with mastitis caused by *S. aureus* during the year 2017. We have followed the same protocol previously described above. The results obtained from these new 26 strains collected during 2017 period showed a new prevalence for all tested antibiotics such as penicillin (27.0%), ampecillin (34.6%), oxacillin (65.4%), amoxicillin associated to clavulanic acid (34.6%) and cefazolin (42.3%). Furthermore, an intermediate resistant rate of 11.5%, 3.8%, 3.8%, 19.2% and 3.8% were found for these already described antibiotics, respectively. Of note, piperacillin antibiotic showed forever a susceptibility of 100.0% against all these new collected strains. The comparison of these new findings with those mainly found in 2003-2005 and 2007-2008 periods are different. As a result, we observed a noticeable reduction especially for penicillin (27.0% in 2017 vs 76.9% in 2003 to 2008 period) and for ampecillin (34.6% in 2017 vs 73.0% in 2003 to 2008 period). This decrease in prevalence of both antibiotics penicillin and ampecillin in 2017 could be associated with veterinarian practices; for example, as of high resistance prevalence of *S. aureus* has emerged against penicicillin and ampecillin many veterinarians avoid their use in treatment of bacterial infections especially mastitis disease caused by *S. aureus* and used other antibiotic families instead Beta-lactams family. As a result, a reduction in prevalence of both antibiotics penicillin and ampecillin has occurred and could be explained by inactivation of some genes (such as *blaZ*, *mecA* and *mecC*) encoding for resistance phenomenon to both prominent antibiotics. However, the new strains showed a resistant prevalence of 65.4% in 2017 instead 3.8% in 2003 to 2008, 34.6% in 2017 instead 0.0% in 2003 to 2008 and 42.3% in 2017 instead 0.0% in 2003 to 2008 for oxacillin, amoxicillin associated with clavulanic acid and cefazolin, respectively. Thus, the manifestation of intermediate resistant strains must be taken into consideration;

because, this phenomenon could be associated to development of genes encoded for these antibiotics such as amoxicillin associated with clavulanic acid, cefazolin or to a progressive inactivation of these genes by their no use by bacteria for several years what makes them unexpressed. Finally, piperacillin antibiotic keeped its effectiveness against older strains collected in 2003 to 2008, and it is also found to be very effective against new strains that have been recently collected in 2017. This needs to reconsider this antibiotic in order to fight and treat *S. aueus* infections originated from bovine mastitis.

We have also grouped our findings from 2003 to 2017 in order to have a global vision on the resistance profiles of *S. aureus* originated from bovine mastitis in the northern west of Portugal. In general, the results obtained from the antimicrobial susceptibility testing for all the tested 52 *S. aureus* isolates showed a prevalence of 57.7% (n=30) (R=52.0%, n=27/52; I=5.7%, n=3/52) of resistant strains to penicillin followed by a prevalence of 53.9% (n=28) (R=52.%, n=27/52; I=1.9%, n=1/52) of resistance to ampicillin and a prevalence of 36.5% (n=19) (R=34.6%, n=18/52; I=1.9%, n=1/52), 26.9% (n=14) (R=17.3%, n=9/52; I=9.6%, n=5/52) and 23.0% (n=12) (R=21.1%, n=11/52; I=1.9%, n=1/52) of resistance to oxacillin, amoxicillin associate to clavulanic acid and to cefazolin, respectively. A very high susceptibility prevalence (100.0%, n=52) was demonstrated for piperacillin. Concerning the results obtained for the *blaZ* and *mecA* resistance genes profiles. Among all tested *S. aureus* isolates (n=52), 67.3% (n=35) were PCR positive for *blaZ* and negative for *mecA* genes, except for isolate 25 that was the only one positive for *mecA*. In addition, the phylogenetic analysis of the *blaZ* gene *S. aureus* isolates consensus sequences collected from 2003 to 2017 were placed in 2 different clades, clade A (cluster A, A.1) and B (cluster B), all closely related to animal and/or human *S. aureus* strains. Isolate 2 appeared in the phylogenetic tree as the most divergent.

Finally, we have indicated in the first part of this thesis that *blaZ* resistance gene plays a role in β-lactam resistance in the tested bovine mastitis *S. aureus* isolates within dairy herds in the northwest of Portugal, especially in case of penicillin and ampecillin antibiotics that have shown a high phenotypic prevalence especially for isolates collected during 2003-2004 and 2007-2008 periods. On the other hand, the findings obtained confirmed that the proportion of *S. aureus* isolates with phenotypic resistance did not match with the proportion of those identified with *blaZ* gene, as isolates with 100.0% of phenotypic susceptibility for all tested antibiotics also harbored *blaZ* gene. We have also found that the antibiotic piperacillin showed high *in vitro* activity against *S. aureus* isolates, suggesting therefore that this could be chosen for *in vivo* treatments instead of penicillin or ampecillin, which is frequently used. We were also able to show diversity inside or between different herds in the northwest of Portugal by means of *blaZ*

phylogenetic analysis from isolates. For this, the evaluation of new bovine mastitis milk samples collected in the same herds, using the same or other methods would be of importance to further discuss the dynamics on resistance patterns of *S. aureus* in the region.

In the first part of this thesis, we have grouped our results from 2003 to 2017 in order to have a global vision on the resistance profiles of *S. aureus* against a set of antibiotics group from beta-lactam family. In the second part of this thesis, we have generalized our investigation to other antibiotics such as gentamicin, tobramycin, amikacin and kanamycin, belonging to Aminosides family; tetracycline from Tetracycline family; erythromycin and clindamycin from Macrolides family; vancomycin from Glycopeptides family and other antibiotics class such as trimethoprim/sulfamethoxazole from Lipopeptides family in order to have also a vision on the resistance profiles of *S. aureus* against these antibiotics from different families more used in veterinary medicine in the northwest of Portugal.

In the literature, the use of antimicrobial agents with narrow broad-specrum in addition to the use of combined antibiotics remained among the new successful strategies followed in the last decades for more effectiveness of these agents against several resistant pathogenic bacteria especially *S. aureus* causing many serious infectious diseases and well-known as one of the most pathogenic bacteria considered to be more resistant among other microbes to Beta-lactams antibiotics family in the most part of the world; but, all these strategies, along with time, showed their limited effectiveness and stayed as temporary solutions in the light of progressive emergence of multidrug-resistant strains. Over decades, many antimicrobial agent families with different mechanisms of action on microbes have been in parallel introduced in order to overcome the resistance problem. For example, family of Aminoglycosides, Tetracyclines, Macrolides Lincosamides, Streptogramins, Glycopeptides, Lipopeptides ... etc. These antibiotics families are extensively used and more described in veterinary medicine for cost-effective prophylactic and therapeutic treatment and they are also used as growth promoters in cattle and poultry. Thus, our main objective in the second part of this thesis was to evaluate in vitro the efficacy of a set of antimicrobials from different antibiotics family to the resistance phenomenon and detection of reliable resistance genes in *S. aureus* isolates originated from bovine mastitis in the northwest of Portugal during the years 2003-2004, 2007-2008 and 2017.

In general, the observed level of antimicrobial resistance findings of the investigated strains in this study was of high value. Thus, the results obtained of antibiotic susceptibility testing demonstrated that the resistance to tetracycline (Tetracycline family) was noted in the 39/52 of isolates (75.0%) while the resistance to erythromycin (Macrolides family) was showed in the 35/52 of isolates (67.3%) followed by a prevalence of 50.0% (26/52), 42.3% (22/52), 36.5%

(19/52), 32.7% (17/52), 28.8% (15/52), 23.1% (12/52), 21.2% (11/52) of resistance to vancomycin, amikacin, kanamycin, clindamycin, gentamycin, tobramycin and trimetoprin/sulfamethoxazole, respectively. Furthermore, intermediate resistance against vancomycin (34.6%, n=18), trimetoprin/sulfamethoxazole (28.8%, n=15), tobramycin (26.9%, n=14), gentamycin (21.2%, n=11), tetracycline (13.5%, n=07), erythromycine (13.5%, n=07), kanamycin (13.5%, n=07), clindamycin (7.7%, n=04) and amikacin (7.7%, n=04) was observed. In parallel, the antimicrobial resistance genes of 33 (63.5%), 30 (57.7%), 16 (30.8%), 10 (19.2%), 08 (15.4%), 03 (5.8%) and 01 (1.9%) strains out of 52 isolates studied were detected positive using PCR by amplification of *ant(4)-I-a, tetM*, *aph(3')-III-a*, *dfrK*, *tetK*, *lnuC* and *spc* genes, respectively. Moreover, the remaining tested genes such as *aac(6)/aph(3)*, *tetK*, *vanA*, *salA*, *vgaC* and *ermT*, were negative. Of note, the no correspondence between genotypic and phenotypic findings has been also observed in this study, especially for aminoglycoside modifying enzymes and for *tet* and *ermT* genes. This no correspondence could be associated to presence of other genes encoding resistance to these antibiotics.

In the second part of this thesis, we have observed that all tested antibiotics in this study showed a high efficacy towards all the *S. aureus* strains collected during the period from 2003 to 2008, whilst, all the strains collected in 2017 demonstrated almost all phenotypic resistance or intermediate against all antibiotics, showing an alarming and dramatic evolution of appearance of new resistant strains. An increasing phenotypic resistance of bovine mastitis *S. aureus* against tested antibiotics, with a high prevalence of phenotypic resistance (≥ 50.0%) among tetracycline, erythromycin and vancomycin was observed. Relatively to tetracycline, erythromycin and for some Aminoglycosides (case of gentamicin, amikacin, kanamycin and tobramycin), Macrolides (case of clindamycin) and for Lipopetides (case of trimethoprim/sulfamethoxazole) obtained results; we suggest that this is an alarming situation and could create a serious challenge to the bovine mastitis therapy in Portugal. Moreover, the *tet(M)*, *aph(3')-IIIa* and *ant(4`)-Ia*, spc genes were the most prevalent antibiotic resistance genes detected.

These findings could be advantageous for design a new specific program for bovine mastitis disease control caused by *S. aureus* in the northwest region of Portugal. Furthermore, also suggests that we must change rapidly our habitual vision and reaction to the better antibiotic to choose and apply for treatment before humanity declares a total capitulation to new infections caused by *S. aureus* in few coming years.

General Perspectives

The results obtained from this study suggest that the use of the Piperacillin antibiotic which has shown high effectiveness, on all strains (collected during 2003 to 2008 and in 2017) that have shown resistance to most of tested antibiotics, can be today a solution to treat certain *S. aureus* infections instead of others tested antibiotics. The performance of antibiogram test has shown that this test can be as key of contraol in order to select the effective antibiotics to treat mammary gland infections caused by *S. aureus*. Thus, it appears that this test remains a definitive and crucial step to make a good use of antibiotics with a more responsibility and with more wise way in order to limit the rapid and dangerous widespread of multi-antimicrobial resistance in North West Portugal. Also, the continuous evaluation of resistance genes acquired by *S. aureus* can help us to discover and understand the different mechanisms of resistance developed by this pathogenic bacteria. However, the realization of the phelogenitic evaluation of *S. aureus* strains can help to obtain precious data on the evolutionary history of these resistant strains, and in particular on their relationship (phylogeny) within the herd in Portugal. The results obtained from this study have shown alarming prevalences mainly with antibiotics from Beta-lactams family (penicillin, ampenicillin… .), Tetracycline family (Tetracycline case), Macrolides family (erythromicine, ...), Aminglycosides family (amikacin, kanamycin), and from Glycopeptide family (vancomycin case). Thus, the use of other alternatives such as the combination of extracts of natural substances can be useful and can be used to treat certain infections caused by multidrugs-resistant strains of *S. aureus*.

In Morocco, it appears from available data on resistance of *S. aureus* to antimicrobials that researches focused or oriented in this context are scarce at national level or absent especially at regional level. Thus, applying equivalent procedures in herds of different Moroccan regions known as high producers of milk could allow to master more antimicrobial resistance phenomenon and by consequent to permit to veterinarians to describe the antimicrobials with more effectiveness in Morocco. In general, this work could be a guide basis for future studies to evaluate and investigate bovine local strains of *S. aureus* and Human *S. aureus* strains of Moroccan origin (Beni mellal; CHR) in order to have a global vision on the resistance profiles of *S. aureus* in Morooco. We propose also:

- the generalization of investigations to other resistance genes developed by *S. aureus* and to test the effectiveness of other antibiotics to fight infections caused by *S. aureus*.

- to study the possibility of inactivation of the genes coded for specific resistance after a long period of non-use of antibiotics which are normally shown to be ineffective because of the development of these specific genes of resistance by *S. aureus*. The fact that our study has shown that there is a remarkable reduction in resistance within strains of *S. aureus* especially for certain antibiotics which are recognized today as outdated antibiotics in the treatment of infections caused by Staphylococci and that these strains have in parallel shown also negative PCR results.

- to test each microorganism able to enter in competing with multiresistant strains of *S. aureus* and assess see if there is an eventual secretion of specific toxins able to destroy multiresistant strains of *S. aureus* by these competitive microorganisms and extracting them for use in the pharmaceutical industry as active molecules for the development of new active antibiotics from molecules secreted by these microorganisms.

- the utilization of natural extracts mixture as potential antibiotics in treatment of infectious diseases.

- the search of more succesful ‘‘effective combination between antibiotics’’ can be useful and can be used to treat certain infections caused by multidrugs-resistant strains of *S. aureus*.

- to study phenotypic and genotypic of antimicrobial resistance developed by other dangerous pathogenic bacteria.

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ANNEX

Columbia C.N.A. Agar Base M560

Columbia C.N.A. Agar Base is used for selective isolation of pathogenic gram-positive cocci from clinical and nonclinical specimens.

Composition**

Ingredients	Gms / Litre
Biopeptone	20.000
Tryptose B #	3.000
Corn starch	1.000
Sodium chloride	5.000
Colistin sulphate	0.010
Nalidixic acid	0.015
Agar	15.000
Final pH (at 25°C)	7.3±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 44.02 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5% v/v sterile, defibrinated blood. Mix well and pour into sterile Petri plates.

Principle And Interpretation

Columbia Agar Base is a nutritionally rich formula containing 5% defibrinated blood, which provides more nutrients and capability of displaying haemolytic reactions. Columbia Blood Agar Base is utilized as a base for preparation of media containing blood and in selective media preparations where various combinations of antimicrobial agents are used as additives. Ellner et al formulated the medium (1) and found that the combination of peptones used gave more rapid and abundant growth of Streptococci, Staphylococci, *Neisseria* and *Haemophilus*

with better-defined haemolytic reactions. Columbia C.N.A. Agar Base is prepared with the same formula as Columbia Agar Base with the addition of 10 mg/litre of colistin and 15 mg/ litre of nalidixic acid to inhibit the growth of gram-negative bacteria and to support the growth of Staphylococci, haemolytic Streptococci and Enterococci when supplemented with 5% blood. Biopeptone and tryptose B supports luxuriant growth of microorganisms and visualization of good haemolytic reactions. Sheep blood allows detection of haemolytic reactions and supplies X-factor necessary for the growth of many bacterial species. Horse blood supplies X-factor and V-factor, therefore is mostly preferred in most laboratories. Yeast extract and cornstarch serve as energy source and neutralizer respectively. It should be noted that this medium has relatively high carbohydrate content and, therefore, beta-hemolytic streptococci may produce a greenish hemolytic reaction that may be mistaken for alpha haemolysis. The addition of the antimicrobial agents, colistin (or polymyxin B) and nalidixic acid, renders the medium selective for gram-positive microorganisms (2). Colistin and nalidixic acid disrupt the cell membrane of gram-negative organisms, whereas nalidixic acid blocks DNA replication in susceptible gram-negative bacteria (3).

Columbia C.N.A. Agar Base with addition of blood gives selective isolation of gram-positive cocci, Staphylococci and Streptococci, particularly when gram-negative bacilli are present and tend to overgrow on conventional blood agar plates. Also used for selective isolation of *Gardnerella vaginalis* . This medium supports growth of *Brucella abortus, Yersinina pestis, Clostridium perfringens* and all commonly occurring *Enterobacteriaceae* without addition of blood.

Quality Control

Appearance
Cream to yellow homogeneous free flowing powder

Gelling
Firm, comparable with 1.5% Agar gel

Colour and Clarity of prepared medium

Basal medium: Yellow coloured clear to slightly opalescent gel. After addition of 5% v/v sterile defibrinated blood: Cherry red coloured opaque gel forms in Petri plates

Reaction
Reaction of 4.4% w/v aqueous solution at 25°C. pH : 7.3±0.2

pH
7.10-7.50
Cultural Response

M560: Cultural characteristics observed with added 5% v/v sterile, defibrinated blood after an incubation at 35-37°C for 40-48 hours.

Organism	Inoculum (CFU)	Growth	Recovery	Haemolysis
Escherichia coli ATCC 25922	>=10^3	inhibited	0%	
Neisseria meningitidis ATCC 13090	>=10^3	inhibited	0%	
Staphylococcus aureus ATCC 25923	50-100	luxuriant	>=50%	beta/gamma
Staphylococcus epidermidis ATCC 12228	50-100	luxuriant	>=50%	gamma
Streptococcus pneumoniae ATCC 6303	50-100	luxuriant	>=50%	alpha
Streptococcus pyogenes ATCC 19615	50-100	luxuriant	>=50%	beta

Storage and Shelf Life

Store the dehydrated and prepared medium at 2 - 8°C. Use before expiry date on the label.

Reference

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