SHORT COMMUNICATION

Two β-lactamics Resistance Genes Detection in Nosocomial Bacteria of Veterinary Interest

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Summary

Nosocomial bacteria acquisition of resistance to multiple antimicrobial agents is a critical concern in medical practice since may result in patient’s increased morbidity and mortality. Additionally, it causes an increased cost to the patient and medical centers as it generates complications and a longer hospital permanency. The information that allows bacteria to develop mechanisms of resistance is found in its genome, having the ability to be transferred in horizontal way to other bacteria. In order to improve pathogen control protocols associated with this type of infection we need to develop a procedure for the dynamic tracing of nosocomial bacterial species, establishing a constant feedback of information regarding their existence, and the characteristics of the environmental responsible for their appearance. This protocol should include identifying the genes responsible for antimicrobial resistance in different species and an analysis of their epidemiological relationship, looking for safeguarding public and animal health.

The objective of this research is to identify the most frequent bacterial genes that confer resistance to the beta-lactam antimicrobials (ampicillin and methicillin). Thus, using a polymerase chain reaction (PCR) assay, we analyzed sixty-seven samples obtained (2007 and 2008) from clinical veterinary centers of the University of Chile. Amplification by PCR detected DNA fragments from the blaTEM gene in Gram-negative and Gram-positive bacteria. Also the mecA gene was detected in Gram-positive bacteria.

Our results, showing a high number of bacteria expressing these genes, should generate great concern to those responsible for the veterinary hospital participating in this study, and strongly suggest that every effort should be made to solve this problem.

Introduction

The use of antimicrobials has had a high impact on medicine. Since the discovery of the first antibiotic (penicillin) and the first chemo-therapeutic molecules (sulfa), these drugs have been used to control infections, thanks to their ability to inhibit or destroy life-threatening bacteria. However, bacteria, through information contained in their genes, possess or have developed different resistance mechanisms against antimicrobials. These mechanisms have limited the effect that antimicrobials produce on bacteria, becoming a very important challenge both for doctors who face complicated treatment diseases, and for pharmaceutical laboratories that must generate more efficient drugs.

The indiscriminate use of antibiotics has not only caused the selection of resistant bacteria in microbial populations, but also causes limitations in antimicrobial therapy. It is for this reason that since the discovery of the first mechanism of resistance to penicillins by Abraham and Chain in 1940, expert scientists have expressed their concern regarding the irrational use of antimicrobials, in order to try to mitigate the selective pressure that fosters the development of mechanisms of resistance and have the greatest amount of therapeutic resources to fight bacteria.

In the hospital enclosures the problem of resistance increases every year, due to the existence of nosocomial bacteria, which correspond to those that are acquired within a hospital premises and whose manifestation occurs 48-72 hours after the patient’s admission, generating a nosocomial infection [1]. These types of infections caused by ubiquitous, opportunistic and highly resistant bacteria have acquired great importance in medical practice and are those that especially affect the immuno-compromised patient, prototype in intensive care units. Thus, the above could explain its high transmission rate.

Nosocomial bacteria generate infections of high morbidity and mortality because they have multiresistance phenotypes. That is, they resist or inhibit the action of two or more antimicrobials, so their treatment and control is very limited. Thus, they affect between 5 and 10% the evolution and recovery of the clinical pictures of hospitalized patients, generating longer residence times in hospital areas and higher treatment costs.

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In this context, antimicrobials belonging to the β-lactam group have been limited in their activity in the treatment of infections due to the increase in resistant bacteria. These antimicrobials are indicated for the treatment of infections caused by susceptible microorganisms, becoming the group of antimicrobials most used in the treatment of infectious diseases, which translates into a major problem in both human public health and animal health. In addition, it has been determined that the factors that have contributed to the increase in nosocomial infections in human medicine hospitals show similarities with those found in veterinary medicine.

Consequently, efforts have been directed to complement the epidemiological surveillance of antimicrobial resistance, using molecular diagnostic techniques, to develop new control strategies. Currently, a large number of very varied studies have been described in human medicine, unlike what happens in veterinary medicine within Chile.

According to the above, in this work and using the Polymerase Chain Reaction (PCR), two genes (meca and blaTEM) involved in the resistance to methicillin and ampicillin antimicrobials were detected in bacteria described as nosocomial. This will allow us to make an approximation of the situation that these genes present in the hospital precincts of the University of Chile and a first approach to the topic in veterinary medicine within the country, in order to develop future epidemiological studies that determine the behavior of these genes in populations bacterial.

**Background**

**Nosocomial infections epidemiological importance**

Infections have different etiological agents: bacteria, viruses, parasites and fungi. In the case of bacteria, they can be transmitted constantly between animals, without necessarily being a health risk. However, in the hospital environment, bacterial populations are established that coexist under a highly variable and demanding environment, which exposes them to various stress situations, which as a selective pressure force, has been the fundamental cause in the appearance of highly bacterial strains resistant [2, 3].

This situation generally occurs under an antimicrobial regimen or invasive procedures with the use of antiseptics for long periods of time. This favors the multiplication of those infectious bacteria, with the genetic-molecular potential to resist antimicrobial therapies, being able to generate serious infections that compromise the survival and recovery of patients due to the nature of the infectious process and its poor response to treatment [4, 5].

The acquisition of multiresistant bacterial agents as commensals is the first stage in the pathogenesis of nosocomial infection. Within this, the host’s immune system plays an essential role, as bacteria must not only use their energy to resist the action of antimicrobials, but also host-specific molecules [6]. Therefore, the acquisition of these infections occurs mainly in immuno-compromised individuals [7].

The U.S. Center for Disease Prevention and Control defines nosocomial infection as an adverse reaction resulting from a systemic or localized condition due to the presence of an infectious agent or its toxin. There should be no evidence that the infectious agent was present or was being incubated before the patient entered the hospital [8].

To be classified as an infection, this condition must manifest itself as a clinical disease and not as a simple colonization, which means that microorganisms are present, but they do not exert adverse effects on the host. However, an asymptomatic patient can be considered infected if pathogenic microorganisms are found somewhere in the body that is normally sterile, such as cerebrospinal fluid or blood [9].

It has been described that more than 70% of the bacteria that cause nosocomial infections are resistant to one or more of the antibiotics commonly used in their treatment. These microorganisms are the cause of an increase in the costs of medical care and hospital stay [10] and can also be transmitted by patients to health care staff, visitors and the community after hospital discharge, with the possibility of causing serious diseases in the community [1, 11].

The incidence of nosocomial infections is very high in a large part of developing countries, due to lack of supervision, the few preventive practices of infections and the high population density that exists in some of the hospitals [12, 13]. Some studies have indicated that nosocomial infections occur in 5% to 10% of all hospitalizations in Europe and North America, and in more than 40% of hospitalizations in different parts of Asia, Latin America and South Africa [14]. In turn, a prevalence survey conducted in 55 hospitals in 14 different countries showed that an average of 8.7% of hospitalized patients present with nosocomial infections [1, 15].

**Nosocomial bacterial agents and clinical considerations**

The bacteria that cause nosocomial infections have undergone major changes over time and initially, the predominant pathogens detected were mainly Gram positive. Then, with the introduction of antibiotics there was a decrease in these infections, becoming mostly produced by Gram negative bacteria [16]. However, in some countries Gram pathogens positives predominate over 60% while Gram negatives do not reach 30% [17-19].

The bacterial agents, described as nosocomial, more abundant -both in human medicine and in veterinary medicine- are the following: *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*Ps. aeruginosa*), *Acinetobacter spp*; *Enterococcus faecium* (*E. faecium*), *Enterococcus faecalis* (*E. faecalis*) and other enterococci: *Enterobacter cloacae* (*E. cloacae*), *Klebsiella pneumoniae*...
All the mentioned bacteria have clinical pictures described: pneumonia associated with mechanical ventilation, blood infections associated with intravenous catheters and urinary tract infections associated with urinary catheters [21]. In addition to these, it has been determined that infections of surgically operated sites are also an important cause of nosocomial infection, and have a high frequency in human hospitals [22]. In the case of veterinary medicine, publications are scarce, but studies reveal results similar to those of human medicine, mentioning urinary tract infections and surgical wound infections as the most important [23-25].

In general, nosocomial bacteria are considered multiresistant microorganisms, that is, they are resistant to two or more antimicrobials. A large number of multiresistant organisms that have epidemiological importance are described. These include: Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE), although their name suggests resistance to only one antimicrobial, they also have resistance to other antimicrobials, so their finding or detection should be a concern constant in hospitals and clinics [26].

Among other bacterial agents that have multiresistance characteristics are: Multiresistant *Streptococcus pneumoniae* (PMS) that resist the action of penicillins and a broad spectrum of antimicrobial agents such as: macrolides and fluoroquinolones; some multiresistant Gram-negative bacilli (BGN-MR) especially producers of extended spectrum betalactamases (BLSEE) (Jacoby and Munoz-Price, 2005), and finally Vancomycin-resistant *S. aureus* (SARV) that have developed great concern in the medical practice [27], since vancomycin is a backup antimicrobial for the treatment of *Staphylococcus aureus* [28].

Thus, among the bacteria with greater clinical-epidemiological importance are the methicillin-resistant *Staphylococcus aureus* (MRSA) and some vancomycin-resistant *Enterococcus* (VRE), which have generated great concern in medical practice. Within *Enterococcus* spp., *E. faecalis* and *E. faecium*, both Gram-positive, are especially important [29]. Finally, among Gram-negative bacteria, *Ps. aeruginosa* has unquestionably developed great importance, and in turn, *Enterobacteria* are still of interest [30].

Considering that nosocomial bacteria are opportunistic, that infect and cause complications of existing pathological processes, they should be addressed strategically. Therefore, it is important that the necessary biosecurity measures are taken for the prevention and control of these bacteria. A fundamental step is to have rigorous control measures regarding the clinical use of antimicrobials and measures that prevent the transmission of pathogens that are already resistant [31].

Without a doubt, prevention is the most important factor in the control of these infections. In spite of the enormous effort made by the medical and healthcare team of the hospitals, to reduce the prevalence of nosocomial infections, it has been seen that they continue to manifest in hospitalized patients and that they can also affect the staff of the place. This has been reflected in the fact that many of the professionals who work in hospitals sometimes act as vectors of the disease, spreading the infection [32-35].

The measures established as part of a control program seek to limit the transmission of microorganisms among patients by means of: 1) proper hand washing practices, use of gloves and masks, 2) isolation strategies, 3) limit the risk of Endogenous infections minimizing invasive procedures and encouraging the appropriate use of antimicrobials and 4) monitoring infections, identifying outbreaks and controlling the responsible agents [36-38].

In general, the hospital medical and healthcare team is recommended to implement a cleaning protocol, considering the frequency of cleaning, types of disinfectants, hand washing practice and the use of aseptic techniques in the management of invasive devices. In addition, protection barriers must be established between animals, decrease hospitalization times and determine possible microorganism vectors [39].

**Bacterial resistance**

**Development and importance of resistance in nosocomial infections**

Historically, bacterial resistance to antimicrobials was defined as persistent bacterial infection, despite the administration of an adequate dose of a specific antimicrobial. Later, this definition was modified by relating resistance to antimicrobial concentration at the site of action. Thus, the resistance could be partial or relative. Bacterial resistance represents the ability of microorganisms to inhibit the action of antimicrobials. Currently, a bacterium is considered resistant when the concentrations of an antimicrobial needed to inhibit its growth in vitro are greater than the concentrations reached in serum or tissues, measured by the minimum inhibitory concentration (MIC) [40].

The information that allows a bacterium to develop a resistance mechanism is found in its genetic material. The emergence and dissemination of resistance mechanisms is favored by the ability of bacteria to modify their genetic material or receiving genetic material horizontally from other bacteria [41].

It is known that every resistance phenomenon has a genetic basis, either by mutations, by acquisition of resistance genes that are transferred between bacteria or by a combination of both mechanisms. Resistance genes may be present in pathogenic, commensal or zoonotic bacteria, but the incidence of these genes varies considerably.
between bacterial species and even among subspecies. For example, Gram-positive bacteria, except for *staphylococci* and *enterococci*, often lack the ability to acquire plasmids that contain resistance genes (R-plasmids) [42]. The presence of a resistance gene does not necessarily lead to treatment failure [43], since the level of expression can be low. Thus, the production of β-lactamase by a bacterium is known, but the development of resistance is dependent on the mode and level of expression of the responsible gene [44, 45].

The widespread use of antimicrobials, irrational and without justification, allows resistant strains of several bacterial species to emerge shortly after use [46]. According to Johnson [47] nosocomial organisms in human hospitals and the pattern of antimicrobial resistance within these establishments change when the use of specific antibiotics is restricted. It is for this reason that it has become necessary to use antimicrobials with a greater criterion, which encompasses both the medical/professional experience, and the theoretical framework in each therapeutic alternative. At present, molecules as novel as moxifloxacin, extended spectrum cephalosporins, carbapenemic and monobactamic derivatives are ineffective when treating bacterial infections, especially nosocomial ones [48, 49].

Antimicrobial resistant microorganisms may also acquire resistance to other antimicrobials that share a mechanism of action. Such relationships, known as cross resistance, occur against agents that are closely related in their chemical formula, such as neomycin-kanamycin. There may also be cross resistance among unrelated chemicals (such as erythromycin-lincomycin). Cross resistance, together with the possibility of acquiring genetic material that confers resistance to more than one type of antimicrobial, enables the appearance of multiresistant bacteria, against which the amount of effective antimicrobials is reduced [50, 51].

Multiple antimicrobial resistance has been demonstrated in nosocomial bacteria in veterinary hospitals and is more frequent in patients who have previously been treated with such antibiotics. In hospitals, where the use of antimicrobials is common, bacteria with antibiotic resistance have a selective advantage compared to other bacterial populations. It is the wide application of antimicrobials in the practice of veterinary medicine, the use of antibiotics in agriculture (antibiotics such as avoparcin or tetracycline) and the use of antiseptics and disinfectants, which would cause this selective pressure on bacteria [52].

The development of bacterial resistance is based, mainly, on the prevalence of resistance genes in bacterial populations and the misuse of antimicrobials, which generate a selective pressure on bacteria with resistance phenotypes. That is, antimicrobial drugs do not generate resistance, but they do favor the survival of resistant microorganisms, by a natural selection process. Generally, this phenomenon occurs in developing countries, where not only antimicrobials are misused but frequently used insufficiently due to financial constraints [1].

Bacterial resistance phenomena not only threaten the success of new antibiotic treatments and the effectiveness of biosecurity measures, they also do so directly affecting the safety of various animal products. Cases of transmission to the human species have been described, from potentially dangerous bacteria, through food and through direct contact [53].

**Mechanisms for transferring genetic Information between bacteria**

In general, there are two different ways of acquiring resistance, intrinsic (vertical) and acquired. Intrinsic resistance to an antimicrobial in some species is of a natural nature, either because they lack cellular mechanisms in which the drug exerts its action or because the bacterial cell wall is impervious to that drug [54].

Acquired resistance, on the other hand, can be caused by mutation or by the transfer of genetic material encoding resistance to a particular antimicrobial. Mutations can be transferred vertically to daughter cells, while horizontal transfer, which allows the transfer of genetic material between bacteria of the same or different species, occurs through the mechanisms of conjugation, transduction and transformation. Within these, conjugation is the most common form of transfer, and consists in the transfer of genetic material thanks to the presence of sexual pilis [55].

Conjugation is a fundamental transfer system for prokaryotes, which allows the transfer of conjugal elements such as plasmids and transposons. These require complex groups of genes that encode the transfer system and subsequent establishment in the recipient cell of said elements. This phenomenon is based on the cellular recognition and establishment of a stable pairing, facilitated by specialized elements, within which a sexual pilus for Gram-negative bacteria and an aggregating substance (SA) of a protein nature in Gram-positive are described. Once this mating is achieved, a conjugative pore is formed, through which single stranded DNA is transferred only or a nucleoproteic complex (relaxoma). The process ends with the synthesis of strands complementary to the single strand in the donor and recipient [56, 57].

The pilis recognize the recipient cell and establish contact by retraction, there are systems without this property, and like Gram-positives they need to collide to mate stably. The SA recognizes the lipoteic acid of the recipient thanks to the fact that it forms a film on the donor surface. In the process, a series of genetic entities can be transferred as conjugative and integron transposons (included in conjugative or mobilizable plasmids) and even the transfer of large genetic segments [58].

The mobilizable elements only encode the genes of the relaxome and not those corresponding to the conjugal
Mechanism of resistance against antimicrobials

In relation to the mechanisms of resistance, from the molecular and biochemical point of view, at least four have been described [62] one bacterial strain being able to use one, several combined or all. These mechanisms are:

A) Inactivation of the drug: this resistance mechanism consists in the production of Enzymes that destroy the antimicrobial by hydrolysis, such as β-lactamas produced by a wide variety of Gram-negative and some Gram-positive bacteria such as staphylococci and streptococci. On the other hand, some bacteria have the ability to produce enzymes that modify the structure of antimicrobials, such as the aminoglycoside modifying enzymes, which have been detected in Gram-negative bacilli of the enterobacteria group.

B) Alteration of the white site of the antimicrobial: this mechanism consists in the modification of the site of action of the drug, so that it is no longer significantly affected by the drug (i.e.: penicillin binding proteins [PBPs]), or, alternatively, the production of said molecule may be amplified to levels such that the dose is insufficient.

C) Active eflux of the drug: it is a special type of exclusion, in which the molecule that initially enters the bacterial cell, through the cell membrane, is transported back to the extracellular environment, thus preventing the molecule from reaching its action site. This mechanism has been described as responsible for tetracycline resistance among Gram-negative bacilli and also in Gram-positive cocci of the genus Staphylococcus.

D) Decrease in the entry of the drug into the bacteria: the bacterial cell can prevent an antimicrobial from reaching an adequate concentration at the site of action, preventing its entry into the intracellular environment, through three mechanisms: (1) Permeability of the outer membrane: clearly defined in Gram-negative microorganisms, which have an outer membrane that constitutes an intrinsic barrier to the penetration of some antimicrobial drugs. (2) Permeability of the inner membrane: another form of resistance of the bacteria consists of an energetic modification that compromises the anionic transporter, which takes the antimicrobial into the cell. The presence of the lipid layer in the membrane acts as a resistance mechanism for hydrophobic drugs, and (3) Porins: they are diffusion channels present in the outer membrane of Gram-positive bacteria. From the modification by mutation of these proteins a decrease in the passage of the antimicrobial is generated.

Antimicrobials

Antimicrobials are natural, semi-synthetic or synthetic chemicals that kill or slow the growth of microorganisms [63]. Antibiotics (from Greek: anti, “against”; bios, “life”) are chemical substances produced by different species of microorganisms (such as bacteria and fungi) or synthesized by laboratory methods, which suppress the growth of other microorganisms and may eventually destroy them. These compounds differ markedly from their physical, chemical and pharmacological properties, as well as in their mechanism of action and antimicrobial spectrum [64].

Mechanisms of action of antimicrobials

They can be basically classified into 5 types: (A) Inhibition of cell wall synthesis: the cell wall provides, among other things, protection against the great osmotic pressure inside the cell, so when it is inhibited or found defective, the cell will suffer lysis. Penicillins and cephalosporins are antimicrobials that use this mechanism, being effective against Gram-positive bacteria due to the high osmotic pressure inside this type of bacteria and having a large proportion of peptidoglycan in the cell wall, unlike Gram-negative bacteria that, in addition if they have low osmotic pressure, they have wall structures that protect them from these antimicrobials. (B) Alteration of the cell membrane: substances that alter this structure, such as polymyxins, modify the permeability, allowing the exit of K ions and macromolecules, with the consequent lycic effect. (C) Inhibition of protein synthesis: for example, aminoglycosides bind to ribosomal subunits which leads to a wrong synthesis of proteins. (D) Alteration of nucleic acid synthesis: they produce inhibition of enzymes that are necessary for DNA replication, recombination and repair. For example, quinolones and fluoroquinolones inhibit the enzyme gyrase, which results in bacterial DNA fragmentation and subsequent cell death, and (E)
Intermediate metabolism inhibitors: such as sulfonamides, which compete with p-aminobenzoic acid, blocking the synthesis of bacterial folic acid, inhibiting the growth and reproduction of the germ [65].

The β-Lactamics

Under this denomination a continuously increasing number of antimicrobials is grouped, whose origin dates back to 1928, when Alexander Fleming discovered a substance capable of inhibiting the growth of *S. aureus*. They form the most used group of clinical antimicrobials, among which penicillins, cephalosporins, carbapenemases and β-lactamase inhibitors, among others [66] Within their molecular structure, they have in common the β-lactam ring, associated with another thiazolidinic ring, forming the 6-aminopenicillanic acid responsible for the activity biological He is associated with a side chain that gives the different antimicrobial and pharmacokinetic characteristics to the different antimicrobial derivatives [67].

The action of β-lactams is developed by inhibiting the synthesis of the bacterial wall, acting in the final stage of the formation of peptidoglycan, called “transpeptidation.” The β-lactams are analogs of a precursor amino acid of the peptidoglycan barrier, D-alanyl-D-alanine, therefore they compete for the active site of an enzyme called “Penicillin Binding Protein” (PLP), which acts at the cross-linking of peptidoglycan. Then the β-lactams covalently bind to the active site of the PLPs, which causes the irreversible inactivation of the enzyme, stopping the formation of peptidoglycan and producing osmotic lysis of the bacterial cell [68].

The introduction of a dimethoxyphenyl group to the side chain of penicillins gave rise to methicillin, which is resistant to enzymatic inactivation produced by β-lactamas of *Staphylococcus aureus*. By adding an amino group to the side chain of the benzylpenicillins, the aminopenicillins were created, as a result of broadening the spectrum of action of the penicillins. Ampicillin, which belongs to this group, is effective against Gram-negative bacteria such as *Escherichia coli* and *Haemophilus influenzae* [69,70].

Bacterial resistance to β-lactam methicillin and ampicillin

The most important mechanism of resistance to β-lactams is the production of β-lactamas, which consist of enzymes that hydrolyse the β-lactam ring of these antimicrobials and convert them into biologically inactive compounds. They are produced by Gram-positive and Gram-negative bacteria (Mediavilla and García-Lobo, 2004) and nowadays a great variety of β-lactamas is known that are divided into four molecular classes: 1) class A, represented by β-TEM type lactamas; 2) class B, represented by rare metalloenzymes; 3) class C, represented by cephalosporinases of enterobacteria, and 4) class D, represented by cloxacillinases.

The *bla*<sub>TEM</sub> gene encodes the most important β-lactamas in the development of resistance to β-lactams. The term TEM was named thanks to Temoniera, a patient from where the first bacterium with β-lactam resistance characteristics was isolated [71, 72] The most characteristic case of resistance by alteration of the sites of action is the resistance to methicillin in *S. aureus*. Methicillin binds with great affinity to *S. aureus* PBP 2 producing lysis of the bacteria. Isolates of methicillin resistant *S. aureus* strains are frequent, which, in addition to their normal PBP2, have a PBP2a, which has very low affinity for methicillin, being resistant to this antimicrobial [73-75] This PBP2a can develop all the required cellular functions but does not allow the binding of β-lactams. Therefore, *S. aureus* methicillin resistant (SAMR) isolates are resistant to all β-lactams and frequently resistant to other classes of antimicrobials. PBP2a is encoded by the *mecA* gene that is found in a long and mobile genetic element called the staphylococcus mec chromosomal cassette (CCS mec) [76].

Epidemiological surveillance of the bacterial resistance

As mentioned earlier, the use of antimicrobials implies the risk of inducing resistance in exposed bacteria, so it is necessary to take the appropriate measures to reduce the occurrence of this phenomenon. However, in order for these measures to be efficient, prior studies are necessary in regard to the type and quantity of antimicrobials used. In addition, the levels of bacterial resistance against specific drugs must be known, characterizing the bacteria and their resistance genes, which would result in the development of a constant surveillance system [77].

One of the international organizations that has taken great care to disseminate the appropriate use of antimicrobials has been the World Health Organization (WHO). In 1997, he made a report delivering a series of recommendations that should meet the countries to control the phenomenon of bacterial resistance in veterinary medicine (WHO, 2003).

In January 1999, the Pan American Health Organization (PAHO) developed a program for Latin American countries, in order to create an updated database to determine the magnitude and impact of antimicrobial resistance, proposing effective and dynamic measures of control, based on accurate data recording and standardized diagnostic procedures by national and international reference laboratories [78].

The WHO project for 2005 incorporated the surveillance of several species of medical interest of which we can mention *Campylobacter* spp., *Uropathogenic E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae* O1 and O139 (enterobacteria). *Neisseria meningitidis*. *Neisseria gonorrhoeae*. *Haemophilus influenzae*. *Streptococcus pneumoniae*. plus β-hemolytic (pathogens acquired in the community); together with *S. aureus*, *Ps. aeruginosa*, *Acinetobacter* spp., *E. faecium*, *E. faecalis* and other enterococci, *E. cloacae*. *K pneumoniae*. *S. marcescens* and *Proteus* spp. (Nosocomial pathogens) [79].
Molecular genetics techniques

The study and monitoring of those genes that give resistance has been carried out thanks to the implementation of molecular genetic techniques. These techniques allow to detect areas of interest in the genome of the microorganism.

Nucleic acid analysis methods have attracted the attention of the scientific world increasingly in recent years and are increasingly used in laboratories as routine diagnostic tests. Among the advantages of these techniques are: they do not need long bacterial isolation processes, are able to detect non-viable organisms, and even find alterations of the genetic material [80].

The Polymerase Chain Reaction (PCR) consists in the in vitro synthesis of a white DNA sequence in a repetitive manner, thanks to the use of splitters (sequence of highly specific nucleotides) that recognize small complementary sequences that flank the segment of the genome to be amplified. This was achieved through the use of Taq polymerase, an enzyme of the Thermophilus aquaticus bacterium that is capable of incorporating free nucleotides at the 3'-end of the splitter, generating copies of the white sequence exponentially, at short intervals of time and at elevated temperatures. It is characterized by its thermostability, high processivity and fidelity [81].

The PCR technique consists of the consecutive repetition of 3 basic steps, which together form a cycle. First, the white DNA is denatured, generating two simple strands. This process is carried out at temperatures that vary between 90 and 96 °C. The second step is hybridization or “annealing” in which the splitters join it. The temperature of this stage is specific to each splitter. The third and final stage is the synthesis of DNA thanks to the action of a thermostable polymerase, which, starting from the starting points, “reads” the target strand, and joins nucleotides that will form the complementary strand to it. The result is two double strands of DNA, made up of the original strand, and the new strand formed, which will be the white strand or template of the next cycle. This process is repeated between 20 and 40 times [82]. At the end of the series of cycles millions of copies of an area of the original DNA have been generated, which are visualized as bands when performing an electrophoresis, in which the DNA is separated according to its molecular weight and its negative electrical charge.

The DNA molecules are deposited in a polyacrylamide or agarose gel immersed in a buffer solution, which is subjected to an electric field. The concentration of the gel determines the density of the solution, and therefore, the speed with which the molecules of interest move. Indeed, the smaller the size of the reaction products, the more concentrated the gel should be used, thus, for diagnostic PCR, 2% agarose concentrations are generally used [83].

Contamination of the sample with external DNA is an important problem when performing the PCR test, since it leads to the amplification of non-specific genetic material that is irrelevant to the test. For this reason, in laboratories where this diagnostic methodology is practiced, precautionary measures must be taken to prevent the entry of contaminating DNA molecules, especially molecules that come from previous experiences to the one being carried out [84].

The detection of antimicrobial resistance genes in endemic strains allows establishing their relationship with mobile genetic elements and the epidemiological relevance that this phenomenon has, considering the mechanisms of genetic recombination that exist [85]. At present, several researchers have followed the antimicrobial resistance genes, determining that the bla TEM and mecA genes have great epidemiological relevance and are detected more frequently in strains with phenotypic resistance characteristics [86-88].

In Chile, there are no investigations aimed at detecting this type of genes in veterinary medicine, and studies carried out by the Institute of Public Health (ISP) and other entities expose an increase in resistance in antimicrobials using classical microbiology techniques [89-90].

In view of the latent risk associated with the transmission of bacteria between humans and animals, the increase in bacterial resistance, the absence of permanent surveillance programs and the high use of antimicrobials in Chile, this study sought to detect the genes that have been described most frequently in human medicine and that give antimicrobial resistance previously. [91-95].

Material and Methods

This research was carried out in the context of FIV project: “Detection of antibiotic and biocidal resistance genes in nosocomial bacteria isolated in the Veterinary Clinical Hospital of the University of Chile”, developed in the Veterinary Microbiology Laboratory, belonging to the Department of Animal Preventive Medicine of the Faculty of Veterinary Sciences of the University of Chile [96-100].

Experimental design

From different units (hospitalization rooms, consultations, surgical pavilions and X-ray rooms) of the veterinary clinical hospitals of the University of Chile, 380 samples were taken from different surfaces (cages, work tables, furniture, anesthesia and x-rays machines) [101-103].

The samples were collected using sterile torulates, which were immediately transferred to the laboratory for processing, according to classical microbiology methodology, which is briefly described below: culture and bacterial isolation by dissemination or depletion, using the clock method, in media of special crops for these purposes. After the incubation of the sown plaques, the morphological and microscopic study of the colonies was carried out, which allowed us to know the type of bacteria developed, (coccus or bacilli, Gram positive or negative), which indicated if the colony was of interest for the study.
If so, it was sown in a special culture medium, to ensure a completely pure development of that strain. Then, it was replicated in semi-transmitted agar (ceparium) and subsequently kept refrigerated until the bacterial strain was identified, using a commercial diagnostic kit (BBL Crystal®). The determination of antimicrobial susceptibility was performed using the Kirby Bauer plaque diffusion method, according to the standards of the National Committee for Clinical Laboratory Standard (NCCLS, 1997), using: Ampicillin (A), Amoxicillin + clavulanic acid (Amc), Oxacillin (Ox), Sulperazone (Sul), Gentamicin (G), Vancomycin (V), Tetracycline (T), Doxycycline (D), Enrofloxacin (Enr), Ciprofloxacin (Cip) and Sulfa/trimethoprim (Stx).

Samples

The bacterial strains of interest for this study met the following requirements: be bacteria described as nosocomial, Gram-positive coccus or Gram-negative bacilli, whose susceptibility profile by the Kirby Bauer method indicated sensitivity, intermediate sensitivity and/or resistance to ampicillin and methicillin and in addition to being multi-resistant.

Sixty-seven bacterial strains were considered nosocomial: Gram-negative (n=28) and Gram-positive (n=39) and were obtained from the veterinary hospitals of the University of Chile (Bilbao and Faculty headquarters) between 2007 and 2008.

Obtaining bacterial DNA

Bacterial DNA extraction was performed using a commercial kit for extraction and purification (Genomic DNA Purification kit, Fermentas®), from cultures of 10^6 CFU/mL, concentration obtained with a McFarlane refractometer. Briefly, at 200 μL of bacterial culture, 400 μL of lysis solution was added, incubated for five minutes at 65 °C, manually homogenizing every 1.5 minutes. Immediately, 600 μL of chloroform was added by gently mixing and inverting five times. It was then centrifuged at 10,000 rpm for two minutes (Heraus Sepatech Biofuge®).

After centrifugation, the upper phase was collected in an Eppendorf tube and 800 μL of precipitation solution was added, mixed gently and centrifuged at 10,000 rpm for two minutes. The pellet obtained was resuspended by the addition of 100 μL of 1.2 M sodium chloride solution. To this mixture, 300 μL of cold ethanol was added and kept at -20 °C for ten minutes. Then, it was centrifuged at 10,000 rpm for four minutes, the supernatant was removed and resuspended in 100 μL of nuclease-free water (Winkler®). Finally, this DNA was used immediately to perform the PCR test or stored at 4 °C for no more than one month.

Gene detection by PCR technique

To carry out the PCR, a Apollo thermocycler (CLP, USA) was used. The references for utilized PCR protocols and primers are summarized in Table 1.

PCR reaction Mix

A 2X PCR Master Mix kit (Fermentas®) was used, which contains thermostable polymerase, deoxynucleotide triphosphates (dNTPs), reaction buffer and MgCl2. In a 0.2 mL Eppendorf tube, 12.5 μL of the Master Mix, 5 μL of each of the splitters and 5 μL of the DNA sample were added, obtaining a total volume of 27.5 μL. Homogenization was carried out using a vortex to ensure reagent mixing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Size (bp), reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>5′-AAAATCGATGGTAAAGGTTGGC-3′, 5′-AGTTCTGCAGTACCGGATTTC-3′</td>
<td>533, Wichelhaus et al., 1999</td>
</tr>
<tr>
<td>Bla TEM</td>
<td>5′-TGCGTCACGAGTTGCTTGAT-3′, 5′-TTATCCGCCTCCATCCAGTC-3′</td>
<td>526, Tenover et al., 1994</td>
</tr>
</tbody>
</table>

Figure 1: Gel de Agarosa al 2%. DNA fragments of 526 pb (blaTEM)
Results

Detection of $\text{bla}_{\text{TEM}}$ gene by conventional PCR in Gram-negative bacteria

The $\text{bla}_{\text{TEM}}$ gene was detected in 2/14 of the sensitive strains, in 1/2 of the strains with intermediate sensitivity and in 8/11 of the resistant strains to ampicillin, determined by the Kirby-Bauer method (Figure 1; Table 1)

Detection of $\text{bla}_{\text{TEM}}$ gene by conventional PCR in Gram-positive bacteria

The $\text{bla}_{\text{TEM}}$ gene was detected in 4/14 of the sensitive strains, in 4/5 of the strains with intermediate sensitivity and in 16/20 of the resistant strains to ampicillin, determined by the Kirby-Bauer method (Figure 1, Table 2).

Detection of $\text{mecA}$ gene by conventional PCR in Gram-positive bacteria

The $\text{mecA}$ gene was detected in 3/32 of the resistant strains and not detected in 2 strains with intermediate sensitivity and neither in 5 sensitive strains to oxacillin, determined by the Kirby-Bauer method (Figure 2, Table 3).

Table 1: Presence of $\text{bla}_{\text{TEM}}$ gene in Gram-negative bacterial strains sorted according to specie and sensitivity to ampicillin

<table>
<thead>
<tr>
<th>#</th>
<th>Specie</th>
<th>A</th>
<th>$\text{bla}_{\text{TEM}}$</th>
<th>#</th>
<th>Specie</th>
<th>A</th>
<th>$\text{bla}_{\text{TEM}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>P. agglomerans</td>
<td>S</td>
<td>+</td>
<td>6</td>
<td>E. cloacae</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>Ps. Aeruginosa</td>
<td>S</td>
<td>+</td>
<td>16</td>
<td>E. cloacae</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>E. coli</td>
<td>IS</td>
<td>+</td>
<td>7</td>
<td>E. coli</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>A. baumannii</td>
<td>R</td>
<td>+</td>
<td>8</td>
<td>E. coli</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>E. cloacae</td>
<td>R</td>
<td>+</td>
<td>10</td>
<td>E. coli</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A: ampicillin; S: sensitive; IS: intermediate sensitivity; R: resistant; (+): gene presence

Table 2: Presence of $\text{bla}_{\text{TEM}}$ gene in Gram-positive bacterial strains sorted according to specie and sensitivity to ampicillin

<table>
<thead>
<tr>
<th>#</th>
<th>Specie</th>
<th>A</th>
<th>$\text{bla}_{\text{TEM}}$</th>
<th>#</th>
<th>Specie</th>
<th>A</th>
<th>$\text{bla}_{\text{TEM}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>E. faecium</td>
<td>S</td>
<td>+</td>
<td>35</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>62</td>
<td>E. faecalis</td>
<td>S</td>
<td>+</td>
<td>36</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>63</td>
<td>E. durans</td>
<td>S</td>
<td>+</td>
<td>37</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>64</td>
<td>E. durans</td>
<td>S</td>
<td>+</td>
<td>38</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>48</td>
<td>E. faecium</td>
<td>IS</td>
<td>+</td>
<td>49</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>53</td>
<td>E. faecium</td>
<td>IS</td>
<td>+</td>
<td>50</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>59</td>
<td>E. faecium</td>
<td>IS</td>
<td>+</td>
<td>51</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>S. kloosi</td>
<td>IS</td>
<td>+</td>
<td>52</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
<td>57</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
<td>67</td>
<td>S. kloosi</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
<td>45</td>
<td>St. Porcinus</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>E. faecium</td>
<td>R</td>
<td>+</td>
<td>46</td>
<td>M. sendentarius</td>
<td>R</td>
<td>+</td>
</tr>
</tbody>
</table>

A: ampicillin; S: sensitive; IS: intermediate sensitivity; R: resistant; (+): gene presence
Discussion

Nosocomial bacteria have generated great concern - both in public and animal health - due to various characteristics: great ubiquity in hospital facilities, invasiveness in immunocompromised patients, high capacity to exchange and acquire genes that provide resistance and also a phenotypic development of multiresistance to different types of drugs, so that their treatment and control every day becomes more complicated (WHO, 2003; Almasaudi, 2018).

The increase in the number of conferences and publications conducted by human doctors and veterinarians in recent times, promoting the rational use of antimicrobials and raising awareness on this issue, is a reflection of the concern generated by infections caused by multiresistant bacteria (Saloojee y Steenhoff, 2001; Sharma et al., 2018). For this reason, it has become necessary to use antimicrobials with a greater criterion, which encompasses both the clinical experience, and the theoretical framework in each therapeutic alternative applied, because currently such novel molecules as moxifloxacin, extended spectrum cephalosporins, Carbapenemic and monobacramic derivatives are ineffectve when treating bacterial infections, especially nosocomial infections (DHQP, 2001; Waheed et al., 2016).

In Chile, there are studies in Human Medicine on this subject (García, 2003), unlike what happens in Veterinary Medicine, where there are no published data (Pinto, 2002). Therefore, it is essential to complete the information we have today in the country and to know the situation of hospital facilities at the University of Chile.

The data obtained in this report after subjecting the different infectious agents described as nosocomial, to the detection of genes that give them antimicrobial resistance, are consistent with those described in the literature consulted (Wichelhaus et al., 1999; Haque et al., 2018).

Thus, after bacterial culture and isolation, the morphological and microscopic study of the colonies, the identification of the bacterial strains and finally the determination of antimicrobial susceptibility of the samples obtained from the Veterinary hospitals of the University of Chile, the strains were selected Bacterial of interest for this study. Observing the results, it is evident and it is important to highlight the high frequency of nosocomial bacteria found in the hospital environment, which have high levels of resistance for most of the studied antimicrobials. This reflects that our country is no stranger to the global problem of increased bacterial resistance, where nosocomial infections occur in 5% to 10% of all hospitalizations in Europe and North America, and in more than 40% of the hospitalizations in different parts of Asia, Latin America and South Africa (Raka et al., 2006; Rajabi et al., 2016; Ekpopphann et al., 2018).

There are many ways in which bacteria can acquire resistance genes, those mechanisms that involve transmissible resistance genes are of particular importance, mainly because they can spread rapidly (Alpuche and Daza, 2002, Sussmann et al., 2003; Bengtsson-Palme et al., 2018). It has been seen that more than 70% of the bacteria that produce nosocomial infections are resistant to one or more of the antibiotics commonly used in their treatment. This would explain the large number of bacterial strains in which we can find resistance genes (Muto et al., 2003; Li and Webster, 2018).

Considering the pressure of selection of the environment, and that a large part of these resistance mechanisms are transmissible among bacteria, it is feasible to think that an increase in the incidence of multiresistant nosocomial bacteria refractory to treatments is predictable (Poole, 2005; Cabrera et al., 2007; Aslam et al., 2018).

Some bacterial strains have intrinsic resistance to certain antimicrobials, others have progressively acquired resistance against others (CDC, 2008), such as vancomycin-resistant Staphylococcus aureus (SARV) that has developed great concern in medical practice, since vancomycin is an antimicrobial of shelter for the treatment of Staphylococcus aureus and there is a possibility that this bacterium has acquired resistance genes from vancomycin-resistant Enterococcus (ERV) (McGuinness et al., 2017)

Therefore, its finding or detection should be a constant concern in hospitals and clinics (CDC, 2008).

The detection of the blaTEM gene in bacteria of the Enterococcus spp genus such as E.faecalis and E.faecium represents a high concern if we consider them to be of great clinical-epidemiological importance (Boerlin et al., 2001; Hanchi et al., 2018) and also have the highest frequency of isolation in the hospital precincts of the University of Chile. Additionally, in E.fecalis the detection of the blaTEM gene was performed in 17 samples, which means 44% of the Gram-positive strains analyzed.

It is very important to relate in a bacterial strain, the presence of a gene that gives resistance, detected by the PCR technique, with the profile of antimicrobial susceptibility
observed by the Kirby Bauer method, either sensitive, with intermediate sensitivity, and/or with resistance to a drug (Brown-Elliott et al., 2012)

In relation to the above, the expected result of this analysis would be to detect a resistance gene, in bacterial strains where resistance is observed for a certain drug by the method of Kirby Bauer, and in the same way, not to detect genes that give resistance, in sensitive strains. However, according to the results of this report, it was observed that a bacterial strain that exhibits resistance against a certain drug by the method of Kirby Bauer, may be negative for the detection of the respective resistance gene. Similarly, the presence of the gene involved in antimicrobial resistance was detected in a sensitive strain or with intermediate sensitivity to a drug (Rasheed et al., 2014; Urbaniak et al., 2018).

This phenomenon could be explained if we consider that the development of resistance is dependent on the mode and level of expression of the responsible gene, determined by the environmental conditions in which the particular bacterial strain is found. In addition, the absence of a gene in a bacterial strain does not limit the development of resistance against a certain drug, since the resistance mechanisms are very numerous and can use one, several combined or all (Chang et al., 2015; Munita and Arias, 2016; Hughes and Andersson, 2017).

In relation to the mecA gene, in this report it was possible to identify three positive bacterial strains, all resistant to oxacillin, a drug similar to methicillin, used to determine antimicrobial susceptibility using the Kirby-Bauer method. Although the incidence of this gene proved to be low compared to the blaTEM gene, its presence is worrying mainly because the mecA gene is in a long and mobile genetic element called Staphylococcus mec chromosomal cassette (CCS mec), which is easily acquired by bacterial strains in the hospital environment (Weese, 2005; Chen et al., 2014; Elhassan et al., 2015).

This suggests that there is a large number of bacteria with these resistance genes and in turn, highlights the concern and interest that should be given to this problem in the near future, mainly due to the high probability that these bacteria act as reservoirs of genes that They provide resistance and may emerge gradually as community pathogens and not only in hospital settings (Duquette and Nuttall, 2004; Beceiro et al., 2013).

A point of interest is the isolated M. sendentarius (46), E. faecium (59) and S. kloosi (67) because they have both the blaTEM gene and the mecA gene, which is possibly due to the ease of bacteria to transfer and acquire genes. In turn, in the hospital environment, bacterial populations are established that coexist under highly variable and demanding conditions, which expose them to various stress situations, which as a selective pressure force, has been the main cause of the emergence of highly resistant bacterial strains (Vali et al., 2008; Guzman-Prieto et al., 2016).

The results obtained suggest that conventional PCR is a technique that has great advantages in the identification of the genes under study. It is a fast method, since the process of obtaining DNA, amplification of the same and electrophoresis can be carried out within 24 hours, which substantially decreases the time it takes to make a diagnosis using traditional methods; direct examination of colonies and culture (Wolcott, 1992; Garibyan and Avashia, 2013). The sensitivity of the method is high, because it allows to detect low concentrations of the DNA that you want to amplify and has a specificity high, especially determined by the sequence of the splitters used and the conditions of the white DNA strand. Additionally, our results are currently being complemented by the subsequent sequencing of the DNA fragments obtained, which will allow obtaining native positive controls (Lecomte et al., 2015; Kamal et al., 2017).

Finally, it is important that for the prevention and control of these bacteria, in the hospital enclosures at the University of Chile, necessary biosecurity measures are taken, where the fundamental steps to follow should be: have rigorous control measures regarding the use of antimicrobials, develop measures that prevent the transmission of pathogens that are already resistant (Alpuche and Daza, 2002; Raasch et al., 2018; ) and finally generate an accurate data record, with the help of standardized diagnostic procedures by national and international reference laboratories (PAHO, 1999) complemented by the active search for the genes responsible for the resistance observed in antibiotics, antimicrobials and biocides in different species, analyzing their epidemiological relationship, in order to try to stop the increase of bacterial strains with multiresistance characteristics, seeking to protect public and animal health.

Conclusion

It was possible to detect the blaTEM and mecA genes, which in nosocomial bacteria give more frequent resistance to ampicillin and methicillin, in samples obtained in veterinary hospitals of the University of Chile (Bilbao and Faculty headquarters) between 2007 and 2008. In particular, the blaTEM gene was detected in 11/28 of the Gram-negative strains and in 24/39 of the Gram-positive bacteria. On the other hand, the mecA gene was detected in 3/39 of the Gram-positive strains.

Finally, the sequencing of the DNA fragments obtained by conventional PCR technique will confirm what is described in the existing literature.

Reference

2. Alpuche C, Daza C (2002) Infecciones nosocomiales por...


17. Center For Disease Control And Prevention (CDC) (2008) CDC/ NHSN Surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. [View Article]


35. Guédon G, Libante V, Coluzzi C, Payot S, Leblond-Bourget...


and cell wall metabolism in Gram-negative bacteria. *Front Microbiol.* 4:128. [View Article]
