Veterinary Sciences and Medicine

SHORT COMMUNICATION

Use of the Polymerase Chain Reaction for the detection of Tetracycline Resistance Genes in Nosocomial Bacteria

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Abstract

Nosocomial infections and antimicrobial resistance represent a worldwide public health problem. Both interact between each other since the bacteria associated to nosocomial infections are usually resistant to antibiotics, being the multiresistance an increasingly frequent phenomenon among involved microbial strains.

In the field of veterinary medicine, one of the most frequently used antimicrobials that has shown a reduced effectiveness is the Tetracycline, a broad-spectrum antibiotic utilized for different therapeutic goals. Resistant bacteria can transmit and acquiring antimicrobial resistant genes, and particularly tetracycline resistant bacteria present a group of genes named *tet*. Currently, there have been 43 *tet* genes described, which code mainly active efflux proteins and ribosomal protective proteins.

The main goal of this work was the detection of three tetracycline resistant genes; *tet*(K), involved in the production of efflux proteins, as well as *tet*(M) and *tet*(O), involved in the production of ribosomal protective proteins, using PCR in previously isolated environmental Gram-positive bacterial strains described as nosocomial from veterinary clinical units of the Universidad de Chile.

The implementation of this molecular biology technic allowed us to identify among phenotypical resistant strains through agar diffusion antibiogram, at least one of the three tetracycline resistance genes. Moreover, in a high percentage of mild resistance strains and in some tetracycline sensible strains, at least one resistance gene was detected.

Introduction

Infectious diseases are currently an important problem worldwide and are undoubtedly one of the main reasons that have motivated research in the biomedical sciences throughout history. Although this great motivation has led the search to resolve, prevent and/or control this pathological state, the human being has also adopted measures of containment such as the creation of hospital facilities and antimicrobial therapy. It is important to note that these advances have allowed benefits to the population, both sick and healthy, and have brought about consequences in relation to the emergence of nosocomial infections and antimicrobial resistance. This is interrelated when considering that the bacteria associated with nosocomial infections are often resistant to antibiotics and particularly to the antibiotics most frequently used in hospital facilities. The factors that contribute to the increase in the incidence of nosocomial infections in human hospitals are beginning to be increasingly common in veterinary medicine. These factors include prolonged hospitalization, management or practices in intensive care units, the use of invasive devices (intravenous and urinary catheter) and the increase in the use of antimicrobial drugs. Thus, the widespread use of antibiotics has led to the selection of resistant bacteria, causing limitations in antimicrobial therapy. In this context, among the antibiotics widely used in veterinary medicine are the tetracyclines, which although they are a family of antibiotics of broad spectrum of action and useful in medical practice, have been limited by the presence of resistant bacteria. They are also capable of transmitting and acquiring antimicrobial resistance genes in a highly effective manner. In consideration of the above, in this Title Report, three tetracycline resistance genes were detected in Gram-positive nosocomial environmental bacteria, isolated in the veterinary hospitals of the University of Chile, contributing to the investigations of nosocomial infections in the veterinary area, with the purpose of having better tools when performing treatments with antimicrobials in patients who resort to veterinary hospital facilities.

Background

Nosocomial infections (from the Greek *nosos*, disease and *komeo*, treat) correspond to those infections that are acquired within a hospital ward and whose manifestation, depending on the incubation period of the infection, can occur 48-72 hours later, or even a once the patient has been discharged [1]. Both nosocomial infections and resistance to antimicrobials are

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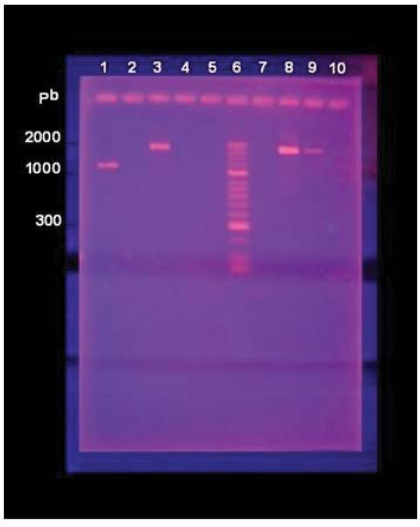
topics that have been intensively studied in human medicine, due to the great problems they generate in hospital facilities. In veterinary medicine, interest in their knowledge has increased in recent years since the practice of empirical treatments persists, without studies of antimicrobial susceptibility, which has meant an increase in the appearance of resistance contributing to the problem already existing in Public Health [2-4]. These infections occur in both developed and other resource-poor countries and are one of the main causes of mortality and morbidity in hospitalized patients, causing high economic costs, where the main factor is the prolonged stay of infected patients. These infections are observed moderately among hospitalized human patients (5-10%), a figure that has not yet been established for veterinary hospitals. However, it is estimated that the factors that contribute to increase the presentation of nosocomial infections are common for both types of patients. The microorganisms that cause nosocomial infections can be transmitted to the community by patients after discharge from hospital, by health care personnel and by visitors [5] (Figure 1).

Resistance to antimicrobials

The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to these compounds from the first moment of use. This is true for agents used in the treatment of bacterial, fungal, parasitic and viral infectious diseases, applying to any disease suffered by any living organism, including humans, animals, fish, plants, insects, etc [6].

The molecular mechanisms of antibiotic resistance have been widely studied and have involved genetic and biochemical investigations from different facets of bacterial cell function. In fact, the study of the action of antibiotics and resistance has contributed significantly to the knowledge of cellular structure and function. Resistance processes are widely distributed in the microbiological realm and have been well described in commensal and pathogenic bacteria. Most of the resistance can be disseminated by one or more gene transfer mechanisms [7, 8].

The resistance to antimicrobials can be considered from two points of view: the biological and the clinical. The sequencing of the complete genomes of dozens of bacteria suggests that all microorganisms have some degree of natural resistance to one or more groups of antimicrobials, either because there are genes that encode resistance mechanisms of greater or lesser effectiveness or because the molecule does not exist white for the action of certain antibiotics. When considering that this natural resistance is predictable (due to the abundant information available about it), its clinical importance is relative, since the clinician relies on it when planning the care of the infected patient. Therefore, acquired resistance is of greater interest because of mutations in genes that the bacterium already has or when it acquires a resistance gene from another microorganism [9, 10].The close contact between pets and humans gives favorable conditions for the transmission of bacteria, either by direct contact (caresses, licking, physical injuries, etc.) or through the domestic environment (contamination of food, furniture, etc.). This situation also occurs in veterinary clinics where a constant relationship is established between patients (pets) and staff. Horizontal transfer of resistance genes can occur in both directions from pets to humans and from humans to pets. For example, human bacteria transmitted to pets can acquire resistance genes from their commensal flora and can be selected by antimicrobial treatments in these animals. Therefore, pets have the potential to contribute to the spread of resistant bacteria acquired to humans and the [11-14]. The predominant role of human activities in the generation of environmental reservoirs of antibiotic resistance has already been accepted. Since 1940, the amount of antibiotics applied to humans released to the environment has been increasing, which provides a sustained selection pressure on populations of resistant strains in all environments. Obtaining an exact



Lane	Sample (content)
1	083 *E. faecium*
2	061 *E. faecium*
3	051 *E. faecium*
4	087 *E. faecium*
5	Negative control (*tet*(M), *tet*(,K))
6	Hyperladder II (50-2000 pb)
7	Control negativo (*tet*(O))
8	520 *E. durans*
9	031 *E. durans*
10	085 *E. faecium*

Figure 1: 2% agarose gel stained with ethidium bromide. Amplicons of 1862 bp tet (M); 1723 bp tet (O);1159 bp tet(K)

quantity of antimicrobials produced by the pharmaceutical industry is difficult, but it can be estimated considering that several million tons of antibiotics have been released into the biosphere during the last half century [15, 16].

Mechanisms of resistance to antibacterial agents

In relation to the mechanisms of resistance, at least four have been described, a bacterial strain being able to use one, several or all of them combined. These recognized mechanisms are: 1) Inactivation of the drug by the production of enzymes that degrade it (eg, beta-lactamases); 2) Modification of the target molecule in such a way that it is no longer significantly affected by the drug (eg, penicillin-binding proteins [PBPs]), or, alternatively, the production of said molecule can be amplified to such levels that the dose is insufficient; 3) Intrinsic exclusion due to the existence of a reduced permeability barrier that prevents the entry of the active compound into the bacterial cell (eg, the lipophilicity of the cell wall of the genus Mycobacterium); 4) Active efflux of the drug 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 medium [17].

Genetics of antibiotic resistance

The discovery of the presence of putative sequences of bacterial genes in eukaryotic genomes has increased the awareness of the great importance of horizontal gene transfer (THG) in the evolution of the genome. Consequently, other aspects of gene transfer have been revealed by the identification and distribution of genomic islands carrying pathogenicity genes and other groups of functional genes of different bacterial genera [18-20].

During therapeutic use, the exposure of bacterial pathogens to high concentrations of antibiotics for prolonged periods generates a severe selection pressure and leads to higher levels of resistance. The way in which a gene from the environment becomes a gene of clinical resistance is not known, but it is obvious that it occurs with some ease [21, 22].

The genetic changes that give bacteria resistance to antibiotics, through the change in their genetic material can be done in two ways:

1) Mutations that modify the preexisting DNA of cells. These alterations involve changes, deletions and investments in DNA bases that involve a change in the genetic material, but do not imply the acquisition of a new DNA to the cellular genome.

2) By acquisition of new genetic material, which involves the capture of new genes within the cell which it expands the genome. This mechanism is the main cause, although not exclusive, of the development of bacterial strains resistant to antibiotics, which cause infections of humans and animals [23, 24].

Transmission and acquisition of resistance genes

Within the mobile genetic elements, we find two types: 1) elements that can move from one bacterial cell to another, which in terms of resistance to antibiotics include resistant plasmids and conjugative resistant transposons and 2) elements that can move from a genetic location to another in the same cell. These last elements include resistant transposons, gene cassettes and ISCR “Insertion sequence common region”. Plasmids and conjugative transposons are transferred from one cell to another by mechanisms that involve replication. Transposons, gene cassettes and ISCR mediate the transfer of genes between sites in the same or different DNA molecules requiring recombination forms, which may or may not include forms of replication. Antibiotic resistance genes accumulate in plasmids because of the activities of these last three recombination systems [25, 26].

Detection of resistance genes

It should be noted that studies of the mechanisms of resistance to antibiotics and their corresponding pathogen gene transfer mechanisms have played a fundamental role in the development of recombinant DNA methods, providing the experimental basis for the modern biotechnology industry [27]. Because of this, genotyping methods for the detection of antibiotic resistance genes are highly required for their speed, precision and sensitivity for the detection of resistance genes in a wide range of pathogenic and commensal bacteria in clinical and environmental samples [28,29] Among the methods of genotyping, used for the detection of antibiotic resistance genes, are the Polymerase Chain Reaction (PCR). This reaction is an in vitro method of DNA synthesis with the that a segment of it is specifically amplified by being delimited by a pair of primers that flank it. Its copying is achieved exponentially through repeated cycles of different periods and incubation temperatures in the presence of a thermostable DNA polymerase enzyme. In this way, millions of copies of the desired DNA sequence are obtained in a matter of hours. PCR is not only an exquisitely specific technique, but also very sensitive, because in principle to practice it, a single molecule would suffice [30, 31].

Tetracyclines

Tetracyclines are a family of antimicrobials with a broad spectrum of action, they exhibit activity against a wide range of Gram-positive, Gram-negative, chlamydial, mycoplasma and rickettsial bacteria, being also used against protozoan parasites. The antimicrobial properties and the absence of adverse effects of these, has allowed its extensive use in the therapy of human and animal infections. These drugs have also been used as a food additive in food animals to act as growth promoters [32-34].

Discovery and development

Tetracyclines constitute a family of natural products, derived from different species of Streptomyces and products that are semi-synthetic derivatives. They were discovered by Duggar in 1940 (Duggar, 1948)35 and began to be used from 1950 to date. Tetracyclines have been classified into three generations, according to the order of discovery, of the pharmacokinetic properties and spectrum of antimicrobial activity. The first

generation are the oldest agents, tetracycline, oxytetracycline, chlortetracycline, chelocardin, demeclocycline, limecycline, metacycline and rolitetracciclina. In the second generation, doxycycline and minocycline are included. And finally, in the third generation, we include tigecycline and new compounds in development such as aminomethylcyclines (BAY73-6944 / PTK-0796) [35-38].

Structure

They are structured by a nucleus of linear tetracyclic conformation, composed of four fused benzene rings to which different radicals can be attached that will give rise to different tetracycline molecules. They all form chelating complexes with different cations, such as calcium, magnesium or iron [39].

Mode of action

The compounds of clinical use have bacteriostatic activity and their mode of action has been well characterized. The antimicrobial function of tetracyclines starts once they enter the cytoplasmic space of bacteria, reversibly binding to the 30S subunit of the ribosome, inhibiting protein synthesis, by blocking the access of aminoacyl-tRNA complexes to the A site of the ribosomal complex [40].

Tetracyclines to interact with their target molecule need to cross one or more membrane systems depending on whether the susceptible organism is a Gram-positive or Gram-negative bacterium [41, 42].

Ribosomal interaction

The tetracycline ribosome interaction has been extensively studied since 1960 through in vitro biochemical analysis. Several studies have indicated a high individual affinity of tetracyclines to the 30S ribosomal subunit, binding dependent proteins S3, S7, S8, S14 and S19, with S7 being the main target identified in the studies and also dependent on the bases of the 16S rRNA , G693, A892, U1052, C1054, G1300 and G1338 that contribute to this close union. But many unanswered questions remain about how tetracyclines prevent the binding of aminoacyl-tRNA to site A or whether it interacts with RNA or ribosome proteins [43, 45].

Tetracycline resistance

The action of tetracyclines of inhibition of protein synthesis, combined with the versatile structure that allows it to traverse the biological membrane with ease, is the key to the broad spectrum of activity of this group of antibiotics. Consequently, its extensive use has led to the selection of resistant organisms involving pathogenic and commensal bacteria, which has severely limited the clinical applications of this [46,47] The interest in establishing the mechanisms of the genetic determinants of resistance and the molecular bases of these mechanisms has been triggered by the increase in bacterial resistance to tetracyclines. The studies are aimed at identifying ways to inhibit tetracycline resistance mechanisms and thus restore the antimicrobial activity of tetracyclines [48, 49].

Resistance to tetracyclines in most bacteria is due to the acquisition of new genes; Genes related to tetracyclines are among the most commonly found resistance determinants among bacteria. They are usually associated with mobile genetic elements, such as plasmids and transposons, which are often conjugative [50].

Mobile genetic elements

Tet genes are often associated with plasmids, transposons, and conjugative transposons which can carry other genes for resistance to antibiotics or heavy metals. The integrons have been identified in Gram-negative genera and in Staphylococcus spp., The tet genes have already been found within integrons [51]. The Tn916-Tn1545 transposons are the most promiscuous family of conjugative transposons described, with a wide variety of hosts including Gram-positive and Gram-negative genera [52].

Currently, in 48 genera of Gram-positive bacteria have been described and determined the mechanisms of resistance to tetracyclines. It is important to mention that not all the tetracycline resistant bacteria have been correlated with the tet genes described so far. A total of 35 genera carry the tet (M) gene, 12 carry the tet (K) gene and 12 carry the tet (O) gene (Roberts, 2010). The tet (K) and tet (L) genes are widely distributed among the Gram-positive species associated with humans, animals and soils and have been found in Mycobacterium, *Nocardia* and *Streptomyces* spp. isolated from patients. The tet gene (M) is often associated with conjugative elements of the Tn916-Tn1545 family. In most Gram-positive species, the tet gene (M) is found in conjugative elements within the bacterial chromosome. The tet (O) gene can be found in conjugative plasmids or in the chromosome, where it is mobile on its own when it is found in conjugative plasmids [53].

In Chile, in the field of veterinary medicine, there are no published records regarding the detection of resistance genes in nosocomial bacteria. This study provides information on the presence of tetracycline resistance genes in environmental Gram-positive bacteria, described as nosocomial and in relation to what is happening in our veterinary hospital facilities, which may even reflect the situation in the country.

Material and Methods

This research was carried out in the context of the project FIV 4602016 and carried out in the Laboratory of Veterinary Microbiology, belonging to the Department of Animal Preventive Medicine of the Faculty of Veterinary and Animal Sciences of the University of Chile.

Samples: 35 Gram-positive coccid bacterial strains were used, obtained in a previous study (Avendaño, 2010), described as nosocomial and phenotypically resistant (R) or with intermediate sensitivity (SI) to tetracyclines determined by Kirby Bauer diffusion antibiogram, in front of a panel of 11 antimicrobials that included tetracycline and doxycycline (Tables 6 and 7).

Obtaining bacterial DNA: To extract the bacterial DNA,

a commercial extraction and purification kit (Genomic DNA Purification Kit, Fermentas®) was used, following the manufacturer's instructions. Thus, 200 µL of a bacterial culture of 1.5 x 10^8 CFU / mL (0.5 of MacFarland) were added 400 µL of lysis solution, incubating at 65° C for five minutes, homogenizing manually every 1.5 minutes. Then, 600 µL of chloroform (Merk®) was added, gently mixing and centrifuged at 10,000 rpm for two minutes (Heraus Sepatech Biofuge®). Then, the upper phase was collected in a 1.5 mL Eppendorf tube and 800 µL of the precipitation solution provided by the kit was added, mixing again and centrifuging at 10,000 rpm for two minutes. The obtained pellet was dissolved in 100 µL of NaCl (1.2 M). To this mixture was added 300 µL of cold ethanol keeping it at -20° C for 10 minutes to precipitate the DNA. Subsequently, it was centrifuged at 10,000 rpm for four minutes, the ethanol was removed, and the obtained pellet was dissolved in 100 µL of nuclease-free water (Winkler®).

Detection of tetracycline resistance genes by PCR: A 96-well 96-well Apollo thermocycler (CLP, USA) was used to perform the Polymerase Chain Reaction and a protocol that included the temperatures, the estimated time for each stage and the number of applicable cycles, according to the gene to detect.

3.1 Primers (Table 1) Genes tet (K), tet (M) and tet (O). The sequence of the primers was selected for its high specificity, extending segments of 1159 bp, 1862 bp and 1723 bp, respectively (Trzcinski *et al.,* 2000).

PCR reaction mixture: A commercial kit (2X PCR Master Mix Fermentas®) was used, which includes thermostable polymerase, deoxynucleotide triphosphates (dNTPs), reaction buffer and MgCl2. 12.5 µL of this Master Mix were deposited in a 0.2 mL Eppendorf tube, together with 5 µL of each of the primers and 5 µL of the hardened DNA sample, obtaining a total volume of 27.5 µL. A vortex was used to ensure the mixing of the reagents. to. DNA amplification It was carried out according to the following protocols (Table 2) (Trzcinski *et al*., 2000).

b. Visualization of the amplified products: It was performed by electrophoresis in 2% agarose gel. Thus, 5 µL of the PCR product was mixed with 1 µL of the commercial loading product. Electrophoresis was carried out at 90 V for 90 minutes. Hiperladder II (Bioscan) was used as molecular weight marker, with DNA fragments between 100 and 2000 bp to detection of the amplified fragments. Then of the electrophoresis, the gel was incubated in ethidium bromide (0.5 µg/mL) (Fermelo ®) and the bands were visualized in an ultraviolet light transilluminator (Transiluminator UVP ®) and photographed with Polaroid ® film.

Biosafety measures: Use of long sleeve apron and latex gloves in visualization of the amplified product involved the use of ethidium bromide and a transilluminator of UV light with an acrylic plate and glasses with UV filter were used. The elimination of the gel incubated in ethidium bromide contemplated its incineration, since the chemical compound has mutagenic properties.

Results

- The tet (K) gene was detected twice; in 2/22 of the strains resistant to tetracycline and doxycycline.
- The tet gene (M) detected in seventeen opportunities; in 10/22 of the strains resistant to tetracycline and doxycycline; 2/4 and 4/7 of the strains with intermediate sensitivity to tetracycline and doxycycline respectively; and in 1/2 of the strains sensitive to doxycycline.
- The tet (O) gene was detected twelve times; in 7/22 of the strains resistant to tetracycline and doxycycline; in 2/4 and 3/7 of the strains with intermediate sensitivity to tetracycline and doxycycline respectively, one of these strains having intermediate sensitivity to tetracycline and doxycycline; in 1/2 of the strains sensitive to doxycycline.
- The tet (K), tet (M) and tet (O) genes were detected simultaneously at one time; in 1/22 of the strains resistant to tetracycline and doxycycline.
- The tet (K) and tet (O) genes were detected simultaneously at one time; in 1/22 of the strains resistant to tetracycline and doxycycline.
- The tet (M) and tet (O) genes were detected simultaneously on six occasions; in 4/22 of the strains resistant to tetracycline and doxycycline; in 1/4 and 1/7 of the strains with intermediate sensitivity to tetracycline and doxycycline respectively.

Discussion

At present, pet ownership has increased considerably and with

Table 1: Primers sequences utilized

Gene	Primers
***tet*(M)**	5'-AGTTTTAGCTCATGTTGATG-3' 5'–TCCGACTATTTAGACGACGG-3'
***tet*(O)**	5′-AGCGTCAAAGGGGAATCACTATCC-3′ 5′-CGGCGGGGTTGGCAAATA-3′
***tet*(K)**	5´-TATTTTGGCTTTGTATTCTTTCAT-3´ 5´-GCTATACCTGTTCCCTCTGATAA-3´

Table 2: PCR protocols by resistance genes detection.

Gen	Cycles	Initial denaturation	Annealing	Elongation	Final elongation	size
***tet*(M)**	35	95º C; 60s	50º C; 60s	72º C; 90s	72º C; 300s	1862 bp
***tet*(O)**						1723 bp
***tet*(K)**						1159 bp

it the concern for the prevention and treatment of the infectious diseases that affect them. As a consequence of these changes, antimicrobial agents are increasingly used in pets, including antimicrobial preparations specifically used in humans. In the clinical practice of small animals, veterinarians usually face economic situations that do not allow the performance of laboratory tests; hence, empirical antimicrobial therapies without susceptibility studies are carried out to ensure good use of these drugs and, on the contrary, contribute to the increase of antimicrobial resistance (Guardabassi *et al.,* 2004).

Thus, antimicrobial resistance is a complex problem involving bacterial species, resistance mechanisms, genetic transfer mechanisms and reservoirs. Several studies have shown that the use of antimicrobials in slaughter animals contributes to the selection of resistant strains and involves risks to humans by the transmission of resistant zoonotic bacteria through the food chain and the indirect transfer of resistance genes from animals to humans. Resistant bacteria can be acquired by man through alternative routes such as person-to-person transmission, environmental exposure and direct exposure to animals (Lloyd, 2007).

It is important to highlight that a large part of the veterinary investigations related to the isolation of nosocomial bacterial strains and their subsequent detection of resistance genes have been carried out based on samples obtained from small animals. In this title report, the samples used were environmental samples from veterinary hospitals, in which bacteria described as nosocomial were detected. The nosocomial bacterial isolates were studied the antimicrobial susceptibility profile and the resistance genes involved in the study were detected, with the intention of obtaining an approximation to the reality in which the veterinary hospitals are in terms of nosocomial infections. When comparing the results of this study with previous research, the important thing to consider is not the origin of the isolates, that is, environmental or commensal, but the antimicrobial resistance patterns and the resistance genes detected in the bacterial species. (Lloyd, 2007; Jackson *et al.,* 2010). Some previous research indicates that nosocomial bacterial isolates that are resistant to tetracyclines also have multi-resistance susceptibility profiles, a situation also observed in this title report, revealing that 65% of the total isolates resistant to tetracyclines (23/35) had multiresistance (Lloyd, 2007; Guardabassi *et al.,* 2004).

Regarding the strains isolated in this work, it agrees with previous studies that show that enterococci and staphylococci are the most interesting in resistant nosocomial veterinary strains (Schwarz *et al*., 1998, Jackson *et al.,* 2010). Jackson in 2009, describes *E. faecium* and *E. faecalis* as the most frequently isolated species of dogs and cats, a situation also found in the present work where 80% (28/35) corresponds to *E. faecium* (Table 3). *Enterococci* have been detected in numerous habitats that include the intestinal tract of mammals, soil, water, plants, insects and food. They are considered leaders of nosocomial infections in humans and are resistant to antimicrobial agents commonly used in hospitals. In addition to being recognized as one of the leading causes of nosocomial infections, they are also considered a reservoir of antimicrobial resistance genes (Jackson *et al.,* 2009). Results from other research indicate that healthy dogs and cats are a source of enterococci resistant to antimicrobials and can act as a reservoir of resistance genes and can be transferred from pets to people, from people to pets and to the environment. In this study the conclusions of these investigations can be corroborated, since the bacterial isolates of this study were obtained from environmental samples

Table 3: Gram-positive bacterial strains according to isolate, species, sensitivity to tetracyclines and doxycyclines and presence of tet (K), tet (M) and tet (O) genes.

Nº	Aislado	Especie	T	D	*tet*(K)	*tet*(M)	*tet*(O)
1	**051.**	*E. faecium*	R	R		+	
2	**076.**	*E. faecium*	R	R		+	
3	**082.**	*E. faecium*	R	R			+
4	**055.**	*E. faecium*	R	R			
5	**083.**	*E. faecium*	R	R	+	+	+
6	**061.**	*E. faecium*	R	R		+	
7	**087.**	*E. faecium*	R	R			
8	**062 s**	*E. faecium*	R	R		+	
9	**511 s**	*E. faecium*	R	R			
10	**054.**	*E. faecium*	R	R			
11	**033-3**	*E. faecium*	SI	R		+	+
12	**341.**	*E. faecium*	R	SI		+	
13	**356 M**	*E. faecium*	R	R			
14	**036 - 1**	*E. faecium*	R	R	+		+
15	**001-3**	*E. faecium*	R	R		+	+
16	**085.**	*E. faecium*	R	R			
17	**358 s**	*E. faecium*	S	SI			
18	**034.**	*E. faecium*	R	SI		+	+
19	**508-2**	*E. faecium*	R	R		+	+
20	**508 M**	*E. faecium*	R	R			
21	**060.**	*E. faecium*	S	R			
22	**538 s**	*E. faecium*	R	R		+	+
23	**317.**	*E. faecium*	SI	S		+	
24	**038 - 2**	*E. faecium*	R	R			
25	**001.**	*E. faecium*	R	SI		+	
26	**003-2**	*E. faecium*	R	R			
27	**014 - 1**	*E. faecium*	R	SI			+
28	**551 - 1**	*E. faecium*	SI	SI			+
29	**520**	*E. durans*	R	S			+
30	**031.**	*E. durans*	R	R		+	+
31	**548 s**	*E. durans*	R	R			
32	**028-1**	*E. durans*	R	S		+	
33	**045-1**	*E. faecalis*	SI	R			
34	**038.**	*S.intermedius*	R	R		+	
35	**078 s**	*S.intermedius*	R	SI		+	

T: Tetracycline; D: Doxycycline; R: Resistance; SI: Intermediate Sensitivity; S: Sensibility. (+): Presence of the gene.

Table 4: Sample number and percentage (%)

	Nº	**%**
Total samples collected	380	100
Strain in study	120	32
Nosocomial strain	89	74.16
Gram + Nosocomial strain	56	62.9
R or IS to tetracycline Gram Positive Strains	**35**	**39.3**

from veterinary hospitals, in which the isolates have a high probability of coming from and have been disseminated by dogs and cats. they entered the hospital ward (Delgado *et al.,* 2007).

Results of this title report indicate that 10/35 (29%) of the strains presented at least one tet gene and possess intermediate sensitivity and / or sensitivity to tetracyclines and/or doxycyclines. The presence of any of the tet genes detected in this study does not imply that this isolate should manifest resistance to tetracyclines, since the presence of one of the genes may exist, but that this is not expressed. For this reason, it should be considered 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 determined bacterial strain is found. On the other hand, in this report in 10/32 (31%) of the strains resistant to tetracyclines and/or doxycyclines the tet genes included in the study were not detected. The absence of the tet genes included in the study does not imply that this isolate should manifest sensitivity to tetracycline, since its intermediate sensitivity or resistance to the antimicrobial may be due to the presence of another tet gene not detected in this memory. This is based on the fact that resistance mechanisms are very numerous and bacteria can use one, several or all of them combined.

All the above reaffirms that the techniques of gene amplification, increasingly used in diagnosis in microbiology, should always be complemented with the antibiotic susceptibility of isolated bacteria, using the Kirby-Bauer method that indicates the resistance pattern or In vitro sensitivity of an organism to a panel of antimicrobials (Woodford and Sundsfjord, 2005). Thus, of the strains resistant phenotypically to tetracyclines and/or doxycyclines, incorporated in this work, 12/35 (34%) did not present any of the three tet genes under study. This can be explained because, although the three tet genes studied are the most frequent, they are not the only ones responsible for resistance to these antimicrobials (Robert, 2010).

Other results of previous investigations that have studied the mechanisms of antimicrobial resistance in enterococci isolated from companion animals, have shown the frequency of the genetic determinants of resistance to tetracyclines, with the tet gene (M) being the most frequent finding followed by tet (O) (Jackson *et al.,* 2010). In this report, the tet gene (M) was identified in 13/33 (39%) and tet (O) was detected in 12/33 (36%) of the enterococcal isolates.

The genetic basis of antimicrobial resistance in *S.intermedius* has been extensively studied among small animal bacteria. Small plasmids mediate resistance to tetracyclines via tet (K). The vast majority of strains of *S.intermedius* seem to prefer resistance genes transmitted by transposons, such as the tetracycline resistance genes tet (M) which is located in conjugative transposons Tn916. In this study in the two isolated strains, the tet (M) gene was detected, which is consistent with previous investigations (Guardabassi *et al.,* 2004).

It is striking that tetracycline-resistant *S.intermedius* contains resistance genes prevalent in resistant enterococci and streptococci (eg tet (M)), suggesting that *S.intermedius* could preferentially acquire these resistance genes from enterococci. The interspecies transfer of aminoglycoside resistance genes transmitted by plasmids has been shown to occur from *E. faecalis* to *S. intermedius* under laboratory conditions. These studies indicate that strains of *S.intermedius* are capable of acquiring resistance genes from enterococci. It is interesting that the research findings can be extrapolated in this report, finding that the strains of *S.intermedius* isolated in this report were obtained from the same sampling areas where E. faecium were also isolated and both isolates showed tet genes (M) (Guardabassi *et al.,* 2004) (Table 5).

In this study, the tet gene (M) was identified in 15/35 (42%) of the isolates. The tet (M) gene is frequently associated with conjugative transposons Tn916 and Tn1545, which explains its ubiquity both in this and in other resistance studies (Chopra and Roberts, 2001, Hegstad *et al.,* 2010)

The manifestation of the bacterial multiresistance corresponds to the expression of the genetic material of the same. In this study, 17/35 (48%) of the isolates showed multiresistance and presence of tetracycline resistance genes, which is expected when considering that these genes are frequently associated with plasmids or conjugative transposons, which carry more of an antimicrobial resistance gene (Hegstad *et al.,* 2010). 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, amplifying it and electrophoresis can be carried out within 24 hours. The sensitivity of the method is high, because it allows detecting low concentrations of the DNA to be amplified and has a high specificity, determined especially by the sequence of the primers used and by the conditions of the white DNA strand. Additionally, the results obtained in this work are being complemented with the sequencing of the resulting amplicons to obtain positive native controls.

Table 5: Nosocomial Gram positive strains distribution, according specie

Strain	**n**
E. faecium	28
E. durans	4
E. faecalis	1
S.intermedius	2
Total	**35**

Table 6: Antimicrobial susceptibility profile to tetracycline of 35 Gram-positive nosocomial bacterial isolates

Nº	Isolate	Specie	Enr	A	Amc	Cip	T	Stx	Van	D	G	Ox	Sul
1	**051.**	E. faecium	R	R	R	R	R	R	S	R	S	R	R
2	**076.**	E. faecium	R	R	R	R	R	R	S	R	S	R	R
3	**082.**	E. faecium	R	R	R	R	R	R	S	R	S	R	R
4	**055.**	E. faecium	R	R	-	R	R	R	S	R	R	R	R
5	**083.**	E. faecium	R	R	-	R	R	R	S	R	R	R	R
6	**061.**	E. faecium	R	R	IS	R	R	R	S	R	S	R	R
7	**087.**	E. faecium	R	R	-	R	R	R	S	R	R	R	-
8	**062 s**	E. faecium	R	R	-	R	R	R	S	R	R	R	-
9	**511 s**	E. faecium	R	IS	-	R	R	R	S	R	R	R	-
10	**054.**	E. faecium	R	R	-	R	R	R	S	R	S	R	-
11	**033-3**	E. faecium	R	R	R	IS	IS	S	R	R	R	R	IS
12	**341.**	E. faecium	IS	R	R	S	R	R	S	IS	S	R	R
13	**356 M**	E. faecium	S	R	R	S	R	S	S	R	S	R	R
14	**036–1**	E. faecium	IS	R	R	IS	R	S	S	R	S	R	R
15	**001-3**	E. faecium	R	R	IS	IS	R	S	S	R	S	R	R
16	**085.**	E. faecium	IS	S	-	R	R	R	S	R	R	R	-
17	**358 s**	E. faecium	IS	R	R	IS	S	R	IS	IS	S	R	R
18	**034.**	E. faecium	IS	R	R	IS	R	S	S	IS	S	R	R
19	**508-2**	E. faecium	R	S	S	R	R	S	S	R	S	R	IS
20	**508 M**	E. faecium	R	S	-	IS	R	R	S	R	S	R	-
21	**060.**	E. faecium	R	IS	-	R	S	S	S	R	S	R	-
22	**538 s**	E. faecium	IS	S	S	IS	R	S	S	R	S	R	R
23	**317.**	E. faecium	S	R	IS	IS	IS	R	S	S	S	R	R
24	**038–2**	E. faecium	IS	S	S	IS	R	S	S	R	S	R	IS
25	**001.**	E. faecium	R	S	S	S	R	S	S	IS	S	R	IS
26	**003-2**	E. faecium	IS	IS	-	IS	R	S	S	R	S	IS	-
27	**014–1**	E. faecium	S	S	S	IS	R	S	S	IS	S	R	IS
28	**551–1**	E. faecium	S	S	-	IS	IS	S	S	IS	R	R	-
29	**520**	E. durans	R	R	-	R	R	R	R	S	S	R	-
30	**031.**	E. durans	S	S	S	S	R	S	S	R	S	R	IS
31	**548 s**	E. durans	S	S	-	S	R	S	S	R	S	IS	S
32	**028-1**	E. durans	S	S	-	S	R	S	S	S	S	S	S
33	**045-1**	E. faecalis	R	S	-	R	IS	R	S	R	R	R	-
34	**038.**	S.intermedius	S	R	IS	S	R	R	S	R	S	R	IS
35	**078 s**	S.intermedius	S	S	S	S	R	S	S	IS	S	S	S

Enr: enrofloxacin; A: ampicillin; **Amc**: amoxicillin + clavulanic acid; Cip:ciprofloxacin; **T**:tetracyclin; **Sxt**: sulfa+trimetropin; **Van**: vancomicin; **D**: doxiciclin; **G**: gentamicin; **Ox**: oxicilin; **Sul**: sulperazone; **R:** resistant; **IS**: intermediate susceptibility; **S:** susceptible

Table 7: Description of hospital place were it obtained samples and tet genes obtained

Nº	NR	ISOLATE	SPECIE	HOSPITAL	SECTOR	Place	GENES *tet*
						Cage	(O)
1	2	**551-1**	E. faecium	**Sta. Rosa**	NH		
2	7	**511 s**	E. faecium	**Sta. Rosa**	NH	Meson	
3	5	**508 M**	E. faecium	**Sta. Rosa**	IH	Meson	
4	5	**508-2**	E. faecium	**Sta. Rosa**	IH	Meson	**(M),(O)**
5	4	**538 s**	E. faecium	**Sta. Rosa**	IH	Meson	**(M),(O)**
6	2	**548 s**	E. durans	**Sta. Rosa**	Surgery – Pavilion	urniture	
7	7	**520**	E. durans	**Sta. Rosa**	Clinic room	Meson	**(O)**
8	9	**076.**	E. faecium	**Bilbao**	IH	Cage	**(M)**
9	7	**054.**	E. faecium	**Bilbao**	IH	Cage	
10	2	**003-2**	E. faecium	**Bilbao**	IH	Furniture	
11	1	**028-1**	E. faecium	**Bilbao**	IH	Furniture	**(M)**
12	9	**055.**	E. faecium	**Bilbao**	IH	Meson	**(M)**
13	3	**001.**	E. faecium	**Bilbao**	IH	Meson	
14	6	**001-3**	E. faecium	**Bilbao**	IH	Meson	**(M),(O)**
15	9	**083.**	E. faecium	**Bilbao**	IH	Meson	**(K),(M),(O)**
16	9	**051.**	E. faecium	**Bilbao**	NH	Cage	**(M)**
17	7	**033-3**	E. faecium	**Bilbao**	NH	Furniture	**(M),(O)**
18	3	**031.**	E. durans	**Bilbao**	NH	Cage	**(M),(O)**
19	1	**078 s**	S.intermedius	**Bilbao**	NH	Cage	**(M)**
20	9	**082.**	E. faecium	**Bilbao**	Surgery	Meson	**(O)**
21	8	**061.**	E. faecium	**Bilbao**	Surgery	Meson	**(M)**
22	8	**062 s**	E. faecium	**Bilbao**	Surgery	Furniture	**(M)**
23	6	**085**	E. faecium	**Bilbao**	Surgery	Machine	
24	6	**045-1**	E. faecalis	**Bilbao**	Rays room	Meson	
25	8	**087.**	E. faecium	**Bilbao**	Rays room	Machine	
26	4	**060**	E. faecium	**Bilbao**	Cat Room	Cage	
27	5	**038.**	S.intermedius	**Bilbao**	Common room	Cage	**(M)**
28	6	**036-1**	E. faecium	**Bilbao**	Common room	Cage	**(K),(M),(O)**
29	5	**034.**	E. faecium	**Bilbao**	Common room	Cage	**(M),(O)**
30	3	**038-2**	E. faecium	**Bilbao**	Common room	Cage	
31	2	**014-1**	E. faecium	**Bilbao**	Common room	Cage	**(O)**
32	4	**317.**	E. faecium	**Bilbao**	Common room	Cage	**(M)**
33	6	**356 M**	E. faecium	**Bilbao**	Common room	Cage	
34	5	**358 s**	E. faecium	**Bilbao**	Common room	Cage	
35	6	**341.**	E. faecium	**Bilbao**	Common room	Meson	**(M)**

AN: Antimicrobial number result from susceptibility assay; **NH:** Non- infectious hospital; **IH:** infectious hospital

Conclusions

The methodology used allowed to detect the three genes most associated with tetracycline resistance in bacteria described as nosocomial. The genes detected are not only present in the bacterial strains defined as resistant to tetracyclines and/or doxycyclines by the Kirby-Bauer method. There is no direct relationship between the result of an antibiogram and the detection of the most frequent resistance genes. The molecular biology technique (PCR) is complementary to the technique of classical microbiology (Kirby-Bauer) in the determination of resistance in a bacterial strain.

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