Positive Controls in the Detection of Genes of Resistance to Tetracyclines in Bacteria of Veterinary Interest

Obreque, B; Jara, M.A; Raggi, L.A; Navarro, C.*

Department of Animal Preventive Medicine. Faculty of Veterinary and Animal Sciences. University of Chile

*Corresponding author: Navarro O Carlos, Department of Animal Preventive Medicine. Faculty of Veterinary and Animal Sciences. University of Chile; E-mail: canavarr@uchile.cl

Abstract
Because the etiologic agents are mainly multi-resistant bacteria, the treatment of nosocomial infections is increasingly complicated. In addition, because bacterial resistance is encoded by genes, it becomes necessary to know and update their frequencies and to guide the control of antimicrobial resistance in hospitals.

Currently, the Polymerase Chain Reaction (PCR) is the molecular tool used for the detection of these genes, but positive controls are needed for the proper interpretation of their results.

Therefore, the objective of this study was to obtain two positive controls for tetracycline resistance genes: tet(A) and tet (B) from Pseudomonas aeruginosa and Pantoeaagglomerans, two bacterial strains resistant to tetracycline. These genes were detected by PCR, sequenced and compared with data from GenBank®.

The results obtained using the Clustal Ω and BLAST program indicated a nucleotide identity percentage (NIP) higher than 90% for tet(B) gene, meanwhile lower nucleotide identity for tet(A) gene is controversial.

Thus, the presence of the tet(B) gene was confirmed in the studied strains and its utilization as positive controls can be suggested. The obtaining of strain that may be used as positive control for tet(A) gene was not achieved, however new primers are proposed.

Keywords: Nosocomial infection, Tetracycline, tet genes.

Background
Nosocomial or intrahospital infections (IN or IIH) have been defined as those infections that are acquired within a hospital and whose manifestation, depending on the incubation period of the infection, can occur 48-72 hours later, or even once given discharge the patient, that is, they are not present or being incubated at the time of admission[1,2]. They are preferentially caused by bacteria, being the most isolated from intensive human units Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis and Acinetobacter baumannii[3,4].

In the case of veterinary medicine, there are few studies carried out in veterinary hospitals where the most prevalent nosocomial agents are isolated and identified. In Chile, some bacteria causing infections in operative wounds have been identified in a hospital of small animals, with Staphylococcus intermedius being the most recurrent agent in both dogs and cats, followed by Actinomycetes pyogenes, Micrococcus spp., and Pseudomonas aeruginosa[5]. We have also identified potentially nosocomial environmental bacteria such as Enterococcus faecium, Enterobacter cloacae, Escherichia coli and Pseudomonas aeruginosa, which presented a high percentage of resistance to 2 or more antimicrobials[6].

To define if a bacterium is resistant to an antimicrobial, it is considered the minimum inhibitory concentration (MIC); when the concentration that the antimicrobial reaches in the tissue does not exceed the MIC, the bacteria have all the possibilities of surviving and it can be indicated that they are resistant[7,8]. This resistance can be a natural property (intrinsic) or the result of a mutation or acquisition of genetic material in the form of plasmids or transposons, through different transfer processes, which is facilitated by the selection pressure generated when using antibacterials[9,10].

Currently, one of the groups of antibacterials that have high levels of bacterial resistance is that of tetracyclines[12-14].


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The main resistance mechanisms described to counteract the effect of these antibacterials are: active expulsion pumps specific for tetracyclines and ribosomal protection proteins, with the enzymatic inactivation of the drug being of less importance[15]. The first mechanism has been described mostly among Gram-negative bacteria, while the second among Gram-positive.

At least, 40 genetic determinants of tetracycline resistance (tet genes) and 3 determinants of resistance to oxytetracycline (otr genes) have been characterized[16]. Most of these genes, including tet(A) and tet(B), encode a cytoplasmic membrane efflux (Tet) protein, which exchanges a proton for a cation-tetracycline complex against the concentration gradient[17]. It is described that these two genes are widely disseminated in nature among Gram-negative bacteria, because they occur mainly in mobile genetic elements[18].

Some studies have evaluated the prevalence of these genes in nosocomial bacteria and it is recognized that the tet gene (B) is the most prevalent in strains of Acinetobacter baumanii[18-20] while tet(A) and tet(B) present a high frequency in strains of Escherichia coli[21]. To achieve this detection, the Polymerase Chain Reaction (PCR) has been used[22]. This technique is based on the exponential amplification of a sequence of interest, in a sensitive and specific way. To perform it, DNA is required, two synthetic oligonucleotides (primers) that give specificity to the technique, the DNA polymerase originally from the bacteria Thermus aquaticus, the four deoxyribonucleotides and a thermal cycler, which allows to vary the temperature according to the stage of development, needing denaturation and extension, temperatures higher than alignment[23,24]. The PCR technique requires positive controls, which are a fundamental element in the interpretation of the results. Currently, in the Microbiology laboratory of the Faculty of Veterinary and Animal Sciences of the University of Chile, there are no native strains that certify the presence of resistance genes that can be used as positive controls, so they must be obtained from phenotypically resistant strains. Due to this deficiency, this study aims to generate native controls, so they must be obtained from phenotypically resistant control strains for the blaTEM genes[25] and mecA genes have been obtained, involved in β-lactam resistance (unpublished data).

These controls will allow confirming the presence or absence of these genes in bacteria isolated from hospital premises, being very useful as a predictive measure for greater control of bacterial resistance, and in the development of a surveillance system that two guide the rotation of antimicrobials to be used, according to the epidemiological situation of each hospital facility[26-28].

Materials and methods

This research was carried out in the Laboratory of Veterinary Bacteriology of the Department of Animal Preventive Medicine in the Faculty of Veterinary and Animal Sciences of the University of Chile.

Two bacterial strains were used: Pseudomonas aeruginosa and Pantoeaagglomerans, isolated and identified in a previous study[20] that showed phenotypic resistance to tetracyclines according to the determination of antimicrobial susceptibility using the Kirby Bauer method and which, when analyzed previously by PCR, amplified bands of size compatible with what has been described for the tet (A) and tet (B) genes[20].

The extraction of the bacterial DNA was carried out using a commercial kit (Genomic DNA Purification kit, Fermentas®), following the manufacturer’s instructions. For its amplification, a commercial kit (2X PCR Master Mix Fermentas®) was used, which contains the thermo stable polymerase, the deoxynucleotide triphosphates (dNTPs), the reaction buffer and MgCl₂.

The amplification conditions of the genes were the same for both tet genes. Briefly, the samples were subjected to 30 cycles, with denaturation at 94°C for one minute, alignment at 55°C for one minute, extension at 72°C for one minute and finally a final elongation at 72°C for five minutes. The used primers are 5’-GTAATTCTGACCTGTCGC-3’ and 5’-CTGCTGG-GAAACATTGCGT-3’ for tet (A) gene and 5’-TTGGTTAGGG-GCAAGTTTGG-3’ and 5’-GTAATGGGCAAATAACCG-3’ for tet (B) gene and the expected band sizes for tet(A) and for tet(B) were 950 bp and 650 bp respectively. The visualization of the PCR products was done by electrophoresis in 2% agarose gel (Winkel®) in Tris acetate EDTA (TAE) buffer (Fermentas®). The PCR product was mixed with a commercial loading product (6X Mass Ruler Loading Dye Solution, Fermentas®) and the electrophoresis was carried out at 90 V for 90 minutes. As a molecular size marker, a standard was used that contains DNA fragments between 100 and 1000 bp (DNA ladder, Fermentas®). After electrophoresis, the gel was immersed in ethidium bromide (0.5 μg/mL, Fer mol®) and the bands were visualized in a transilluminator of ultraviolet light (Transiluminator UVP®) and photographed with a digital camera.

The biosafety measures included the use of sterile material, the use of long-sleeved apron and gloves. The use of a transilluminator of UV light and ethidium bromide, contemplated the use of an acrylic plate and glasses with filter, as well as the incineration of the gels.

The sequencing of the resulting DNA fragments was carried out by the Genytec Company (Genetics and Technology Ltd.). Subsequently, these sequences were aligned using the Clustal Ω online program (free access), obtaining a consensus sequence for each gene and each strain, comparing them with some described in the GenBank® and finally determining the nucleotide identity percentage (NIP) for the genes of interest[21]. The criterion for classifying the tet genes was based on that previously used, considering at least one NIP ≥ 80% to be classified within any of the genetic determinants already described[22].

Results

Detection and sequencing of tet(A) and tet(B) genes in bacteria described as nosocomial.

In figure 1, the bands obtained when performing PCR are visualized: 300 bp bands for the gene tet(A) and 650 bp for the tet (B) genes.
The detection of tet(A) and tet(B) genes in two bacterial strains

Figure 1: PCR detection of tet(A) and tet(B) genes in two bacterial.

Table 1: Consensus sequences for BOR1 and BOR2 (tet(B)) and BOR3 and BOR4 (tet(A)) in two bacterial strains

When carrying out the multiple alignments for the tet(B) gene values greater than 90% were found for both consensus sequences: BOR1 (P. agglomerans) and BOR2 (P. aeruginosa). While for tet(A) the sequences BOR3 (P. agglomerans) and BOR4 (P. aeruginosa) reached lower and controversial PIN values (Table 2).
The described methodology allowed obtaining ampli-
cons of sizes close to 650 bp for the tet(B) gene and 300 bp for the tet(A) gene from samples from P. agglomerans and P. aeruginosa, two environmental bacterial strains described as po-
tentially nosocomial. Obtaining amplicons of 650 bp was a first sign of the presence of the tet(B) gene. However, the presence of 300 bp amplicons with respect to the tet(A) gene generated an unresolved question, because these same strains were previously analyzed by PCR, amplifying 950 bp bands. The nucleotide se-
quences obtained allowed us to demonstrate the presence of the tet(B) gene and definitively rule out the alternative that the 300 bp fragment is part of the tet(A) gene, because despite reaching a relatively high PIN value (83%) in P. aeruginosa, its alignment with the other sequences described was not homogeneous, a sit-
tuation that is even more evident in the strain of P. agglomerans. This result would not correspond to a failure in the extraction of bacterial DNA, which is corroborated by the presence of ampli-
cons of 650 bp; nor would it be attributable to the primers, who were prepared again to avoid confusion; The reaction mixture was the same, therefore it does not represent an error either and finally, the program introduced in the thermo cycler is the same for both PCR protocols. Therefore, for the detection of this gene soon, it is advisable to use alternative primers, either previously used or designed by computer programs (in silico) using as reference sequences already described and published in gene banks.

When obtaining the nucleotide sequences and perform-
ing the alignment through the Clustal Ω program, the obtaining of two positive control strains for tet(B): P. agglomerans and P. aeruginosa was corroborated, since the NIP value reached (≥ 93%) allows classify them in this genetic determinant, surpass-
ing the NIP value of 80%, recommended in the case of tet genes. However, the question remains about the presence of the tet(A) gene in the analyzed samples.

In relation to this, when incorporating the 300 bp se-
quence in the database of the free access on-line program called BLAST[36], the sequence BOR4 (P. aeruginosa) shows a 93% nucleotide identity with a segment of the complete genome of P. aeruginosa, which was expected. However, by incorporating the sequence BOR3 (P. agglomerans), the program finds no iden-
tity in its vast nucleotide collection, suggesting perhaps non-spe-
cific amplification. For this reason, it would be interesting to re-
iterate the use of the in silico designed starters proposed in this study, to corroborate the obtaining of fragments compatible with the expected size and verify their identity through sequencing, evaluating their possible usefulness as positive controls.

Finally, the verification of the presence of the tet(B) gene in P.agglomerans and P. aeruginosa is-accentually- a find-
ing because this gene has not been previously described in any of these strains.

Conclusions

The methodology described allowed to obtain two native control strains: P. agglomerans and P. aeruginosa, to continue with the study of the tet(B) gene, one of the 40 genes involved in tetracycline resistance and constitutes a first step towards the study of the relationships between antimicrobial susceptibility deter-
mined by the Kirby Bauer method and the effective presence of a gene involved in resistance.
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