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

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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 nucleotidic identity percentage (NIP) higher than 90% for *tet*(B) gene, meanwhile lower nucleotidic 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 baumanni*[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 *Actinomyces 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[9-11].

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 *tetgene* (B) is the most prevalent in strains of *Acinectobacerbaumanni*[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 deoxynucleotides and a thermal cycler, which allows to vary the temperature according to the stage in 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 positive controls useful in the detection of the *tet*(A) and *tet*(B) genes, complementing the investigations in which positive control strains for the bla_{TEM}[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[29] 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[30].

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 $MgCl_2$.

The amplification conditions of the genes were the same for both *tet* genes. Briefly, the samples were subjected to 30 cycles, with denaturation at 94ºC for one minute, alignment at 55ºC for one minute, extension at 72ºC for one minute and finally a final elongation at 72ºC for five minutes. The used primers are 5'-GTAATTCTGAGCACTGTCGC-3' and 5'-CTGCCTGGACAACATTGCTT-3' for *tet* (A) gene and 5'-TTGGTTAGGGGCAAGTTTTG-3' and 5'-GTAATGGGCCAATAACACCG-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 (Winkler®) 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, Fermelo®) and the bands were visualized in a transilluminator of ultraviolet light (Transiluminator UVP®) and photographed with a digital camera.

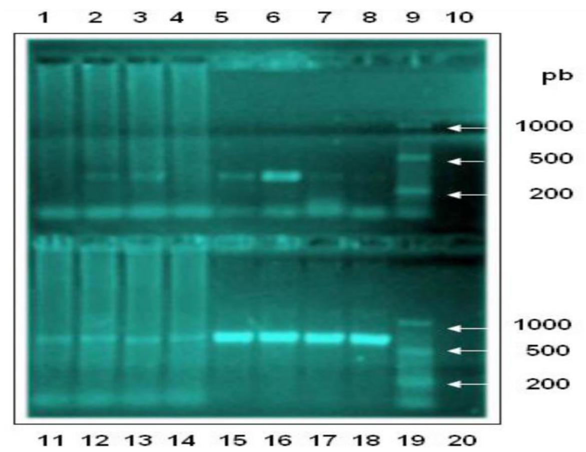
The biosafety measures included the use of sterile material, the use of long-sleeved apron and gloves. The use of a transilluminator of UV light and ethidium bromide, contemplated the use of an acrylic plate and glasses with filter, as well as the incineration of the gels.

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[31]. 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[32].

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 *tet*(A) detection
Lanes 1-4: P. agglomerans
Lanes 5-8: P.aeruginosa
Lane 9: Marker
Lane 10: negative control
The *tet*(B) detection
Lanes 11-14: P. agglomerans
Lanes 15-18: P.aeruginosa
Lane 19: Marker
Lane 20: Negative control
Marker: 100-100pb Negative control: canine herpes DNA

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

Determination of nucleotidic identity percentage (NIP) with respect to GenBank®.
Once all these fragments were sequenced, a consensus sequence was obtained for each bacterial strain and tet gene studied (Table 1).

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

BOR1: consensus sequence for *tet*(B) (*P.agglomerans*) TTATGTTTTGGGTTCCGTGAAACCAAAAAATACACGTGA-TAATACAGATACCGGAAGTAGGGTTGAGACGCAATCGAAT-TCGGTATACATCACTTTATTTAAAACGATGCCCCATTTTGTT-GATTATTTATTTTTCAGCGCAATTGATAGGCCAAATTCCCG-CAACGGTGTGGGTGCTATTTACCGAAAATCGTTTTGGAT-GGAATAGCATGATGGTTGGCTTTTCATTAGCGGGTCTTG-GTCTTTTACACTCAGTATTCCAAGCCTTTGTGGCAG-GAAGAATAGCCACTAAATGGGGCGAAAAAACGGCAGTACT-GCTCGGATTTATTGCAGATAGTAGTGCATTTGCCTTTTTAG-CGTTTATATCTGAAGGTTGGTTAGTTTTCCCTGTTATAATA-ATATTGGCTGGTGGTGGGATCGCTTTACCTGCATTACAG-GGAGTGATGTCTATCCAAACAAAGAGTCATCGCAAGGT-GCTTTACAGGGATTATTGGGAGCCTTACCAATGCACT	BOR2: secuencia de consenso *tet*(B) (*P.aeruginosa*) TGCCTTGGTTAAAGCGGGGCCTATTATTGGTGGTTTT-GAGGAGAGTTTTTACCGCATAGTCCCTTTTTTTATC-GCTGCGTTGCTAAATATTGTCGCTTTCCTTGTGGTTAT-GTTTATTGTTCCGTGAAACCAAAAATACACCGTGATAA-TACAGATACCGAAGTAGGGGTTGAGACGCAATCGAATTC-GGTATACATCACTTTATTTAAAACGATGCCCATTTTGTT-GATTATTTATTTTTCAGCGCAATTGATAGGCCAAATTC-CCGCAACGGTGTGGGTGCTATTTACCGAAAATCGTTTTG-GATGGAATAGCATGATGGTTGGCTTTTCATTAGCGG-GTCTTGGTCTTTTACACTCAGTATTCCAAGCCTTTGTGG-CAAGAAGAATAGCCACTAAATGGGGCGAAAAAACGG-CAGTACTGCTCGGATTTATTGCAGATAGTAGTGCATTTG-CCTTTTTAGCGTTTATATCTGAAGGTTGGGTGAGGTTTGTC-CCTGTTTTTAAATTTTATTA
BOR3: secuencia de consenso *tet*(A). (*P.agglomerans*) CCGTTACACGGCTTCGTCGGGCTCTAGGACCCTCCGGGC-GTCGTCGAGGACCCGCACGTTGCCCCCGTCCACCCGCGC-CCGTTCGACGAGTTCCAGGAGCCGCTCCTGAAGCTTTTTC-GATCTCGTTGGTCAAGATGTCCCCGATGTGCCGGTTGAG-CCCCTTGCTTTCCTTGCCCTCCACGCCCACCGCGGTCAG-GCCCTTGACGCGTTGGGCGATTCGTGCCATCCCCTGTG-GCTCCTGTTGCACATGGTACACGGAATCAATGTTATCCAT-GCTGTCCAGGCAGA	BOR4: secuencia de consenso *tet*(A) (*P.aeruginosa*) TGTAATTCTTGTAATTGTGAGCAGGGTCGCCGCGTCA-CAGGTTTCGTCGAGGCTCAGGGCCAGCCGCCGGTCGT-CAAAAAACGCGCAGGTTGACCCGTGCCGCCCGCGC-CCGTTCGAGGATCGCCGCCTGACGCTCGCCGACCTCGTA-GAACATGGTGTCGAAGAAGTGCCGAAAAAAGCCGCCG-CAGTCCCTTGCTTTCCAGGCCCGCCGCCAGCAGCGCGGT-CAGGCGCTGGACGCGTTGGGCGATGCGTTTCAGGTCCTGG-GCCCAGGAGACGGCATACAA

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).

Table 2: Nucleotidic identity percentage (NIP) for BOR1, BOR2, BOR3 and BOR4 by Blast Program

BOR1

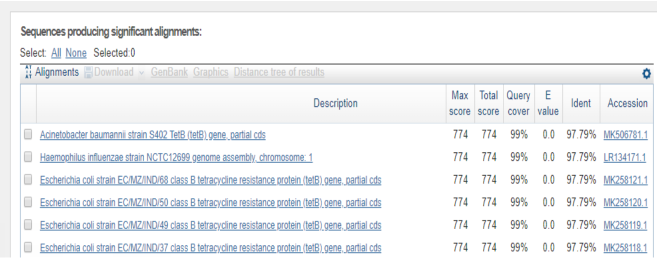
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Acinetobacter baumannii strain TG60155 chromosome	898	898	99%	0.0	98.44%	CP036283.1
Escherichia coli strain A68 TetB (tetB) gene, partial cds	898	898	99%	0.0	98.44%	MK532895.1
Escherichia coli strain A5 TetB (tetB) gene, partial cds	898	898	99%	0.0	98.44%	MK532892.1
Escherichia coli strain A3 TetB (tetB) gene, partial cds	898	898	99%	0.0	98.44%	MK532890.1
Acinetobacter baumannii strain VB31459 plasmid unnamed1, complete sequence	898	898	99%	0.0	98.44%	CP035931.1
Acinetobacter baumannii strain S402 TetB (tetB) gene, partial cds	898	898	99%	0.0	98.44%	MK506781.1

BOR2



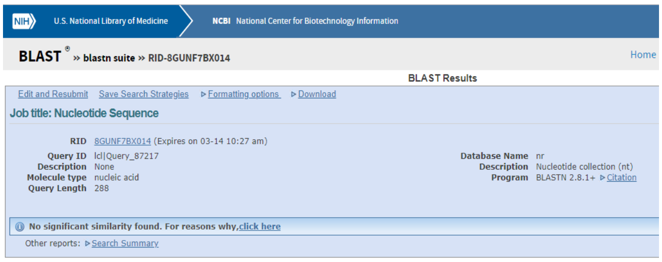
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Acinetobacter baumannii strain S402 TetB (tetB) gene, partial cds	774	774	99%	0.0	97.79%	MK506781.1
Haemophilus influenzae strain NCTC12699 genome assembly, chromosome: 1	774	774	99%	0.0	97.79%	LR134171.1
Escherichia coli strain EC/MZ/IND/68 class B tetracycline resistance protein (tetB) gene, partial cds	774	774	99%	0.0	97.79%	MK258121.1
Escherichia coli strain EC/MZ/IND/50 class B tetracycline resistance protein (tetB) gene, partial cds	774	774	99%	0.0	97.79%	MK258120.1
Escherichia coli strain EC/MZ/IND/49 class B tetracycline resistance protein (tetB) gene, partial cds	774	774	99%	0.0	97.79%	MK258119.1
Escherichia coli strain EC/MZ/IND/37 class B tetracycline resistance protein (tetB) gene, partial cds	774	774	99%	0.0	97.79%	MK258118.1

BOR3



NIH U.S. National Library of Medicine NCBI National Center for Biotechnology Information

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BLAST Results

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Job title: Nucleotide Sequence

RID 8GUNF7BX014 (Expires on 03-14 10:27 am)

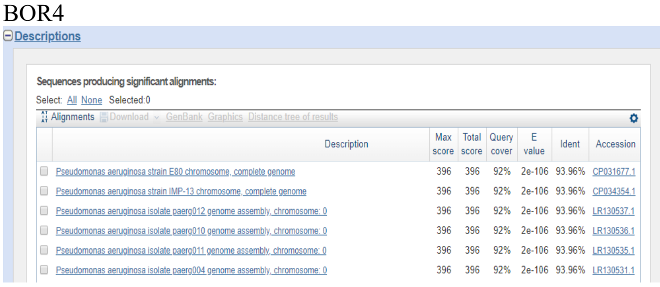
Query ID lcl|Query_87217
Description None
Molecule type nucleic acid
Query Length 288

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.1+ Citation

No significant similarity found. For reasons why, click here

Other reports: Search Summary

BOR4



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas aeruginosa strain E80 chromosome, complete genome	396	396	92%	2e-106	93.96%	CP031677.1
Pseudomonas aeruginosa strain IMP-13 chromosome, complete genome	396	396	92%	2e-106	93.96%	CP034354.1
Pseudomonas aeruginosa isolate paerg012 genome assembly, chromosome: 0	396	396	92%	2e-106	93.96%	LR130537.1
Pseudomonas aeruginosa isolate paerg010 genome assembly, chromosome: 0	396	396	92%	2e-106	93.96%	LR130536.1
Pseudomonas aeruginosa isolate paerg011 genome assembly, chromosome: 0	396	396	92%	2e-106	93.96%	LR130535.1
Pseudomonas aeruginosa isolate paerg004 genome assembly, chromosome: 0	396	396	92%	2e-106	93.96%	LR130531.1

Discussion

Nowadays, it is necessary to have tools to guide the control of bacterial resistance in hospitals. One of these tools could be based on the detection of resistance genes, which would allow a better choice of antimicrobials to be used, complementing the information obtained in the antibiogram.

For this molecular detection, an alternative is to use the PCR technique. An important risk in its realization is the contamination with exogenous DNA, which may come from mainly from previous reactions or using contaminated reagents. To avoid this, it is recommended to respect the work rules, use reagents of certified quality, disposable material and have a physical space separate from areas where other activities are carried out[33,34].

The described methodology allowed obtaining amplicons 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 potentially 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 sequences 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 situation 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 amplicons 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[35] or designed by computer programs (*in silico*) using as reference sequences already described and published in gene banks.

When obtaining the nucleotide sequences and performing 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, surpassing 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 sequence 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 identity in its vast nucleotide collection, suggesting perhaps non-specific amplification. For this reason, it would be interesting to reiterate 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 -accordingly- a finding 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 determined by the Kirby Bauer method and the effective presence of a gene involved in resistance.

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