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Full Length Research Paper

Tetracycline resistance genes in *Pasteurella multocida* isolates from bovine, ovine, caprine and swine pneumonic lungs originated from different Greek prefectures

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The aim of the present work was to analyze the antibiotic resistance of *Pasteurella multocida* isolates from sheep, goats, cattle and pigs originated from eight different Greek prefectures, to determine the class(es) of the *tet* genes, to investigate any possible association with plasmids or/and chromosomes and to study the geographical distribution of the resistant isolates. Eighteen out to one hundred isolates were resistant to tetracycline with a minimal inhibitory concentration (MIC) higher than 128 µg/ml. The *tet*H gene was found in thirteen (72.2%) and the *tet*B gene was found in four isolates (22.2%). After treatment with the restriction enzymes *Eco*RV, *Eco*RI, *Pst*I and *Bsp*HI, the *tet*B gene showed a new polymorphism. Partial sequencing of the *tet*H and *tet*B genes revealed five polymorphisms in the *tet*B gene and two polymorphisms in the *tet*H gene; phylogenetic and molecular evolutionary analyses were also conducted. Both *tet*B and *tet*H genes revealed patterns clearly differentiated from the rest of *Pasteurella* species meaning that the genes could be transferred from a non *Pasteurella* species. The results presented provide further knowledge and evidence of the role played by commensals as of resistance determinants.

Key words: *Pasteurella multocida*, *tet* genes, multiple sequence analysis.

INTRODUCTION

Pasteurella multocida causes economically important diseases in food-producing animals and exhibits resistance to a large number of antimicrobial agents; as a result a limited number of antimicrobial agents is available for treatment including, sulphonamides, tetracycline and streptomycin. The use of tetracycline, for both the treatment and the prevention of diseases in which *P. multocida* is involved, results in high percentages of tetracycline-resistant strains (Kehrenberg

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and Schwarz, 2000). Resistance to antimicrobial agents, including tetracycline depends on the host, the geographical origin and the antimicrobial pre-treatment. Antimicrobial resistance has been reported in a wide variety of animal pathogens for example *P. multocida* from cattle; (McEwen and Fedorka-Cray, 2002). Data collected from 13 European countries showed that isolates of *P. multocida* were resistant to different anti-microbial agents in eight countries. From 1987 to 2004, the percentage of tetracycline resistant isolates from cattle in different European countries ranged between 1% in England and 24% in Italy (Lizarazo et al., 2006; Kaspar et al., 2007; Wallmann et al., 2007; Hendriksen et al., 2008). In Greece, all *P. multocida* strains isolated from pigs and rabbits in 1984 to 1987 were susceptible to tetracycline (Ekateriniadou, 1989).

In the present study, the *tet*B*, tet*A*, tet*M and *tet*H genes were studied. The t*et*B gene which is part of the non-conjugative transposon Tn*10*, has been found in genus *Pasteurella* (Kehrenberg et al., 2001) and it is the most frequently isolated *tet* gene in *Enterobacteriaceae*. The t*et*A gene, encoded also by the transposon Tn*10*, is one of the most widespread *tet* genes in *Enterobacteriaceae* (Kehrenberg et al., 2001, 2005; Millan et al., 2009) found in a variety of ecosystems including estuarine waters (Henriques et al., 2008). The *tet*M gene is the most prevalent determinant in *Listeria* species (Chen et al., 2010) and common gene conferring tetracycline resistance in *Staphylococcus aureus* http://jac.oxfordjournals.org/contnent/64/3/490.full and widely distributed among both Gram-positive and Gram-negative bacteria; this is probably due to the association of *tet*M gene with integrative and conjugative transposons as Tn*916* which can be expressed both in Gram-positive and Gram-negative bacteria (de Vriest et al., 2009). The tetM gene has been detected in the chromosomal deoxyribonucleic acid (DNA) of *P. multocida* isolates. The *tet*H gene which seems to be indigenous to *Pasteurellae* was detected on plasmids pVM111, pMHT1, pPMT1 and pPAT1 and on the transposon Tn5706 (Hansen et al., 1993; Kehrenberg et al., 1998, 2001). The aim of our study was to determine the class(es) of the *tet* genes present in *P. multocida* isolates, to identify their association with plasmid or/and chromosome and to investigate the geographical distribution of the resistant isolates in Greece.

MATERIALS AND METHODS

Bacterial isolates and antimicrobial susceptibility testing

One hundred *P. multocida* strains were isolated from pneumonic tissue samples that originated from sheep, goats, cattle and pigs originated from eight different Greek prefectures. The samples were inoculated on blood agar plates (5% v/v sheep blood) and were aerobically incubated at 37°C for 18 to 24 h. After isolation, microscopic examination and biochemical testing (oxidase, catalase and indole production) were used for the final identification of the strains (Garity et al., 2004). The determination of tetracycline resistance was performed by the agar diffusion method using disks of tetracycline (30 µg/ml) and the phenotypic resistance of tetracycline was further analyzed by determining the MIC according to the CLSI' broth micro dilution procedure, using two-fold dilution series in the range of 8 to 128 µg/ml.

Identification of the *tet* gene classes

Both genomic and plasmid DNA extractions were carried out: genomic DNA extraction was performed in the tetracycline resistance isolates using the PureLink ^{TM} Genomic DNA Kit (Invitrogen) while plasmid DNA extraction was performed using the PureLink ^{TM} HiPure Plasmid DNA Purification Kit (Invitrogen). For the evaluation of the method applied in this study concerning the accuracy of the plasmid DNA isolation and particularly whether fragments of chromosomal DNA might be isolated with plasmids, closed circular plasmid DNA from the tetracycline-resistant *P. multocida* strains was purified also by equilibrium centrifugation in CsCI-ethidium bromide gradients (Sambrook et al., 2001). The purified plasmids were used to transform competent *Escherichia coli* (XL1-Blue) cells, which subsequently were plated in LB agar containing 50 µg/ml of tetracycline. The appearance of tetracycline-resistant strains in all cases, confirms the presence of tetracycline-resistant genes in the examined plasmids. Further, in the above plasmids derived from the *P. multocida* strains were detected with Polymerase chain reaction (PCR) the tetB, tetH and tetH genes, respectively, as in the method applied in the present study. Additionally, for the evaluation of chromosomal DNA isolation and particularly whether large plasmids might be segregated with chromosomal DNA, plasmid curing was applied in two tetracycline-resistant *P. multocida* strains (706/14 and 806/8 p) by growing the bacteria on Brain Heart infusion agar in the presence of 0.002% sodium dodecyl sulphate (SDS) and incubation for 24 h, at 42°C (Sivropoulou et al., 2000). Plasmid isolation from totally 30 colonies of *P. multocida* 706/14 p, revealed that two were free from plasmids, and from 45 colonies from *P. multocida* 806/8 p, five were free from plasmids. Since the above colonies were remained resistant to tetracycline, the tetracycline resistant genes are located in the chromosome as detected with the method applied in the present study.

For PCR analysis six class-specific primmer sets were used to amplify the *tet*A, *tet*B, *tet*H and *tet*M genes in all the resistant isolates (Table 1).

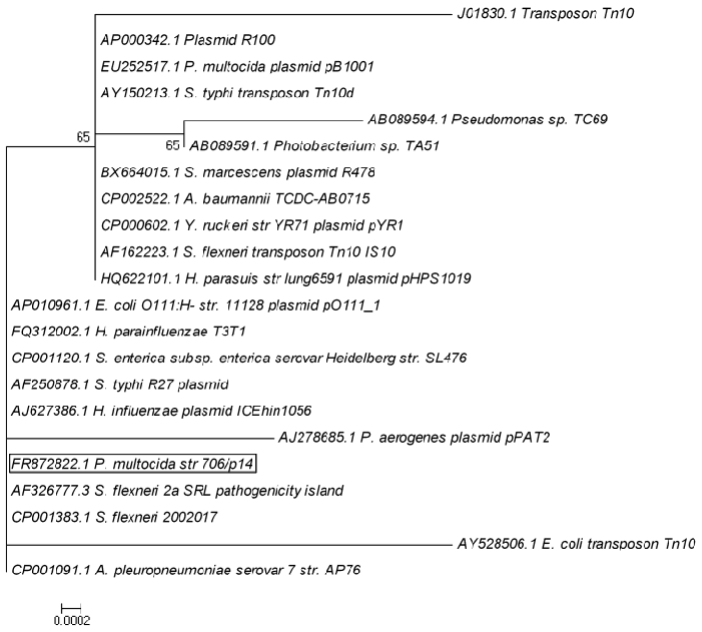
In order to optimize the methodology, three different PCR protocols were used: (a) a short protocol (SP) described by Koike et al. (2007), (b) a two-temperature step-down protocol (TSP) characterized by a higher sensitivity described by Aminov et al. (2002) and (c) a long time protocol (LTP) carried out to enhance a longer part of the *tet*B and *tet*H genes (Hansen et al., 1993; Kehrenberg and Schwarz, 2000). Amplicons of the *tet*B and *tet*H genes were of 1170 and 1076 bp, respectively. DNA amplifications using the LTP protocol were carried out for DNA analysis by the restriction fragment length polymorphism (RFLP) method as well as by sequencing (Fluit et al., 2001). DNA amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research). Non-amplified isolates by the SP protocol were further analyzed by the TSP protocol.

Analysis of the *tetB* and *tetH* classes

The isolates carrying the *tet*B and the *tet*H genes were treated with the restriction enzymes *Ecor*V*, Ecor*I*, Pst*I*, BspH*I to detect possible polymorphisms. Reactions were performed in a total volume of 15 µl according to the manufacturer's instructions. The resulting fragments were separated by 3% low melt agarose gel

Table 1. Primers used for the identification of *tet*A, *tet*B, *tet*H, *tet*M genes of *P. multocida* isolates.

Primer set	Sequence (5'→3')	Annealing temperature (°C)	Protocol	Fragment size (bp)
*tet*A	F′ GCGCGATCTGGTTCACTCG R′ AGTCGACAGYRGCGCCGGC	61	SP-STP	164
*tet*B	F′ TACGTGAATTTATTGCTTCGG R′ ATACAGCATCCAAAGCGCAC	61	SP-STP	206
*tet*M	F′ ACAGAAAGCTTATTATATAAC R′ TGGCGTGTCTATGATGTTCAC	58	SP-STP	170
*tet*H	F′ CAGTGAAAATTCACTGGCAAC R′ ATCCAAAGTGTGGTTGAGAAT	61	SP-STP	185
*tet*B	F′ ACGTTACTCGATGCCAT R′ AGCACTTGTCTCCTGTT	55	LTP	1170
*tet*H	F′ ATACTGCTGATCACCCGT R′ TCCCAATAAGCGACGCT	55	LTP	1076



The *tet*B gene of this study is enclosed within the box. The gene from the Greek isolate is clustered separately from other species of the *Pasteurella* genus

Figure 2. Phylogenetic tree of *tet*B gene.

electrophoresis (UltraPure™ LMP Agarose, Invitrogen).

Sequencing analysis of the *tet*B and *tet*H genes

The t*et*B and *tet*H genes were partially amplified using the LTP protocol and the primers *tet*HLTPF, *tet*HLTPR and *tet*BLTPF, *tet*BLTPR. Sequencing analysis was performed on a Sequence Scanner version 1.0 (Applied Biosystems) and EditSeq, MegAlign modules of the Lasergene Ver.7.1 software (DNASTAR Inc., Madison, WI, USA). Complementary alignments were also performed using ClustalW2 multiple sequence alignment software (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). The *tet*B gene was aligned with the corresponding sequences from the bacteria *Haemophilus parainfluenzae*, *Haemophilus influenzae, Pasteurella aerogenes*, *Pasteurella multocida* and from *plasmid R100.* The sequence of the *tet*B gene in the *Tn10* transposon element (J01830.1, Genbank) was used as reference sequence (Figure 2). The *tet*H gene was aligned with the corresponding sequences from two different strains of *P. multocida*, *P. haemolitica*, *Actinobacillus pleuropneumoniae*,

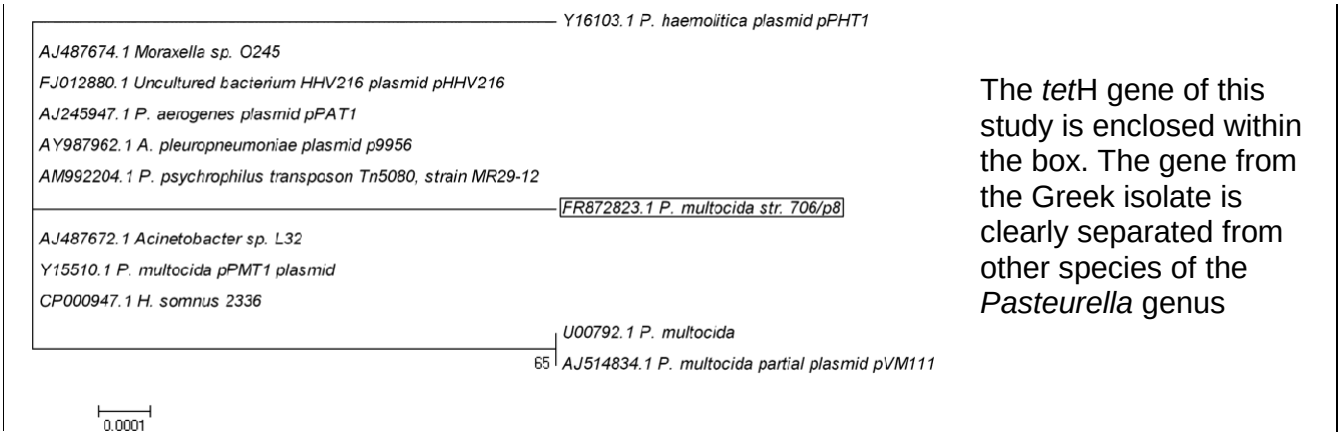


Figure 3. Phylogenetic tree of *tet*H gene.

Table 2. Polymorphisms in *tet*B gene.

Accession number		Nucleotide position					
		511b	842b	902b	988b	1014b	1060b
J01830.1	nt	ACT	GAA	GAT	GAG	TTG	ACT
	aa	T	E	D	E	L	T
706/p14tetB	nt	GCT	GGA	GTT	CAG	TTG	GCT
	aa	A	G	V	Q	L	A
FQ312002.1	nt	GCT	GGA	GTT	CAG	TTG	GCT
	aa	A	G	V	Q	L	A
AJ627386.1	nt	GCT	GGA	GTT	CAG	TTG	GCT
	aa	A	G	V	Q	L	A
AJ278685.1	nt	GCT	GGA	GTT	CAG	TTT	GCT
	aa	A	G	V	Q	F	A
AP000342.1	nt	ACT	GGA	GTT	CAG	TTG	GCT
	aa	T	G	V	Q	L	A
EU252517.1	nt	ACT	GGA	GTT	CAG	TTG	GCT
	aa	T	G	V	Q	L	A

*Numbering is according to the highlighted is the reference sequence, nt-nucleotide sequence, aa-amino acid sequence.

and *P. aerogenes.* Plasmid PVM111 was used as reference sequence (Figure 3). Both amino acid sequences were aligned with the corresponding sequences (Tables 2 and 3). The amino acid sequence of the *tet*B gene in the *Tn10* transposon element was used as reference sequence.

Nucleotide sequence accession numbers

The nucleotide sequence of the 1069 bp segment of the *tet*B gene and the 987 bp segment of the *tet*H gene were deposited in the EMBL database (accession no FR872822 and FR872823, respectively).

RESULTS

From the one hundred *P. multocida* isolates, eighteen showed resistance to tetracycline with MIC higher than 128 µg/ml. The origin of the isolates is showed in Figure 1. The size of the amplicons for each of the three PCR protocols used in the study is shown in Table 1. The *tet*H gene was found in thirteen (72,2%) and the *tet*B gene in four isolates (22,2%). In one isolate (5,5%) none of the four *tet* genes was detected. Both genes were found in chromosomal as well as in plasmid DNA. Only one

Table 3. Polymorphisms in *tet*H gene.

Accession number		Nucleotide position		
		410b	755b	765b
AJ514834.1	nt	GTT	GCG	GGT
	aa	V	A	G
706/p8 tetH	nt	GCT	GCG	GGG
	aa	A	A	G
U00792.1	nt	GTT	GCG	GGT
	aa	V	A	G
Y16103.1	nt	GCT	GGG	GGT
	aa	A	G	G
Y15510.1	nt	GCT	GCG	GGT
	aa	A	A	G
AY987962.1	nt	GCT	GCG	GGT
	aa	A	A	G
AJ245947.1	nt	GCT	GCG	GGT
	aa	A	A	G

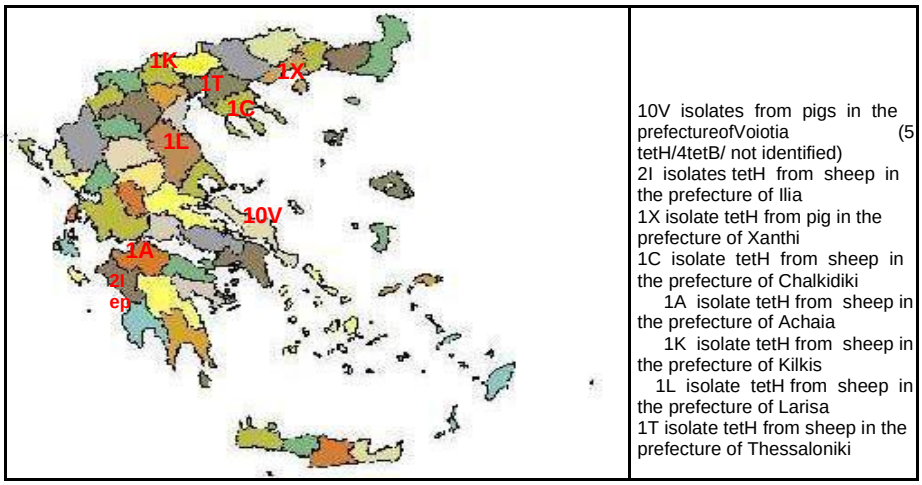


Figure 1. Geographical distribution of the local isolates.

isolate was found carrying the *tet*H gene in plasmid but not in chromosomal DNA. Amplified *tet*B and *tet*H genes were analyzed by the RFLP method. Digestion was performed with *EcoR*V, *EcoR*I, *Pst*I and *BspH*I restriction enzymes. The amplicons of *tet*B gene resulted to the same RFLPs patterns as the reference sequence of Transposon Tn10 (J01830, Genbank). Only treatment with *BspHI* resulted in a different profile: in contrast to the local isolates, the reference strain showed a recognition site for the enzyme *BspH*I in position 951/955. No polymorphisms were identified in the amplicons of the *tet*H gene.

Amplicons of the *tet*B and *tet*H genes were partially (1060 bp and 987, respectively) sequenced. Unique *tet*B and *tet*H sequences were identified. Analysis of the *tet*B gene and comparison with the corresponding sequences (Table 2), revealed five Single Nucleotide Polymorphisms (SNPs) (A538G, A892G, A929T, G1038C, A1087G). Polymorphism A538G could be detected after digestion with the restriction enzyme *Tsp45*I (GTSAC). From the analysis of the *tet*H gene two polymorphisms were found (C460T, G815T). Polymorphism C460T was common

(Table 3) while polymorphism G815T was found for the first time. Both amino acid sequences were aligned with the corresponding sequences. Comparison between the *tet*B and transposon Tn*10*, revealed five SNPs (T171A, E281G, D301V, E330Q, T354A). Polymorphism T171A was common among the *tet*B amino acid sequences of *H. parainfluenzae, H. influenzae*, and *P. aerogenes*. The other four polymorphisms (E281G, D301V, E330Q and T354A) were common not only among *H. parainfluenzae*, *H. influenzae* and *P. aerogenes* but also among Plasmid *R100* and *P. multocida*. These four polymorphisms have not been found in *Tn10* transposon element. One polymorphism (V137A) was found in the *tet*H aminoacid sequence. Polymorphism V137A was common among strains of *P. haemolytica*, *P. multocida*, *A. pleuropneumoniae* and *P. aerogenes*. The phylogenetic trees for both genes were created using the Neighbor-Joining method with bootstrap test (500 replicates). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Based on the *tet*B dendrogram (Figure 2) we can observe the close clustering of the Greek *P. multocida* isolates with species of *Shigella, Salmonella, Haemophilus* and *Escerichia*. The Greek *P. multocida* isolates of the *tet*B gene were less homologous to those of *P. aerogenes* plasmid pPAT2 and also clustered separately from the *tet*B of *P. multocida* plasmid pB1001. As shown in the dendrogram (Figure 3), the *tet*H gene of the Greek *P. multocida* isolates has evolved and separated from the cluster of *P. multocida* pPMT1 plasmid (Y15510.1), *P. aerogenes* plasmid pPAT1, *Acinetobacter* sp. and *Haemophilus somnus*. Furthermore, the *tet*H gene of *P. haemolytica* plasmid pPHT1 has followed an evolutionary process that differentiated it from the cluster forming a distinct branch. *P. multocida* U00792.1 and *P. multocida* partial plasmid pVM111, depicted in a common branch, also evolved separately from the rest of the entries.

DISCUSSION

Antimicrobial resistance is a concern for animal health but little is known about the magnitude of this problem as the surveillance of resistance in exclusive animal pathogens (for example, *Moraxella bovis*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*) is poor compared with surveillance of enteric bacteria. Knowledge and control of resistance in commensals is important as they can act as reservoirs of resistance determinants. The flora of the upper respiratory system of animals that have been treated with antimicrobial agents can also serve as a reservoir of resistance factors. Tetracycline is the most frequently used antimicrobial agents in veterinary medicine. Antimicrobial resistance of *P. multocida* strains has been reported in a wide variety of animal pathogens (McEwen and Fedorka-Cray, 2002; Milan et al., 2009). Data collected from 13 European countries showed that isolates of *P. multocida* from eight countries were resistant to different antimicrobial agents including tetracycline (Kaspar et al., 2007; Hendriksen et al., 2008).

It is important that 18% of the strains isolated from clinically healthy animals in Greece showed resistance to tetracycline higher than 128 µg/ml. The significant increase of the tetracycline resistant isolates contributes to the selection and spread of resistance in animals not only by the movement of carrier animals between herds but also by the assembly of susceptible animals in close confinement, and the movement of resistance determinants throughout the ecosystem (McEwen and Fedorka-Cray, 2002; Adelowo and Fagade, 2009). Moreover, the spread of *tet* genes is often facilitated by their location on mobile genetic elements, such as plasmids and transposons. For the first time in Greece, *tet* genes were investigated. The presence of the tetH and *tet*B genes in both genomic and plasmid DNA, suggests that they are carried on transposable elements as previous studies have shown for example, *tet*B gene with the transposon element Tn*10* (Kehrenberg et al., 2001) and *tet*H gene with the transposon element Tn*5706* (Kehrenberg et al., 1998). The *tet*H gene was found in the majority of the tetracycline resistant *P. multocida* isolates, which is not surprising as it is considered to be indigenous for the genus *Pasteurella* (Hansen et al., 1993) and it has been detected on plasmids pVM111, pMHT1, pPMT1 and pPAT1 and on the transposon Tn*5706* (Hansen et al., 1993; Kehrenberg et al., 1998, 2001).

Similarly, the *tetB* gene is the most frequently observed *tet* gene among *Enterobacteriaceae*, as part of the non-conjugative transposon Tn*10* (Chalmers et al., 2000). The wide distribution of the *tet*B across Gram–negative genera, including *Escherichia, Enterobacter*, *Proteus, Salmonella, Actinobacillus, Haemophilus, Moraxella*, and *Treponema* indicates a great likelihood of horizontal gene transfer occurrence of tetracycline resistance (Speer et al., 1992; Roberts, 1996). Moreover, the *tet*B gene was first detected in *H. influenzae*, a member of the *Pasteurelllaceae* commonly isolated from the lungs. Most of the polymorphisms can be detected in other strains or species (Tables 1 and 2). Glycine (255G) of the *tet*H gene might have a substantial role in the functionality of the protein, thus, the observed mutation was synonymous. Perhaps, this is why no mutations have been previously described at this site. Based on the dendrogram, the *tet*B gene in the Greek isolates of *P. multocida* is more homologous to species other than *Pasteurellae* leading us in two explanations: the gene could probably has been transferred to the isolates from a non *Pasteurella* species or it is still in an ancestral form which has not been altered as it has in other species of its genus, thus clustering separately from them. The local

(Greek) *P. multocida tet*H gene revealed a different pattern of clustering. Nevertheless, as in the case of *tet*B, the local isolates *tet*H gene are clearly differentiated from the rest of *Pasteurella* species.

Finally, the absence of *tet*A and *tet*M genes suggests that a wide spectrum of *tet* genes should be used in future studies. Although our results are similar to those of other researchers (Claudio et al., 2003), further research on other genes involved in tetracycline resistance, such as *tet*D, *tet*G*, tet*L and *tet*O, would provide a more comprehensive view of the tetracycline resistance scheme among Greek *P. multocida* isolates. Moreover, recent data support the premise that the treatment of infected animals has to be based on local knowledge and the observed resistance patterns. In the future, data on the prevalence of resistance should be used to develop guidelines for appropriate antimicrobial use in veterinary medicine.

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