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Genotypic and phenotypic tetracycline-based properties of *Trueperella pyogenes* isolates from bovine samples

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Abstract

The purpose of this study was to investigate the tetracycline resistance in *Trueperella pyogenes* isolates from bovine samples in Burdur, Turkiye, and assess 16 tetracycline-resistance genes distribution among the isolates. Forty-nine *T. pyogenes* isolates were phenotypically characterized for anti-microbial resistance to doxycycline, oxytetracycline and tetracycline by disc diffusion method. Presence of tetracycline genes of *T. pyogenes* was investigated by multiplex and singleplex polymerase chain reaction. Our results indicated that 87.80% and 42.86% of the isolates were resistant to tetracycline and oxytetracycline, respectively, and the rate of resistance to doxycycline was 6.12%. Total of 21 (42.85%) were carrying tetracycline-resistance genes and *tet*(A) was present in 12 (24.49%) isolates; whereas, the *tet*(W) gene was identified in 9 (18.37%) and 2 (4.08%) of the isolates carried both *tet*(A) and *tet*(W), respectively. The study indicated antibiotic resistance patterns of tetracycline agents and links to the tet-genes among *T. pyogenes* were detected. It makes it worthwhile that this is the first report for detection of *tet*(A) gene in *T. pyogenes*.

Introduction

Trueperella pyogenes (formerly *Arcanobacterium pyogenes*) most frequently occurs as a commensal and opportunistic pathogen of the gastrointestinal system as well as upper respiratory and urogenital tracts mucosae of domestic and wild animals.[1,2] The *T. pyogenes* is a common causative agent of mastitis, metritis, abscesses, pneumonia, arthritis, endocarditis and osteomyelitis.[3-7] Moreover, recent decreases in milk production being associated with mastitis, as well as causing reproductive losses and reductions in meat quality have increased interest in understanding the effects of pathogenic *T. pyogenes*.[8,9] Among humans, sporadic cases of *T. pyogenes* infections have been reported in patients who have been in contact with animals.[10-12]

Traditional antibiotics such as beta-lactams have been administered for a long time in veterinary medicine to treat *T. pyogenes*.[9,13] These situations can be often explained by the fact that there is limited information about the *in vitro* susceptibility of *T. pyogenes* against anti-microbials.[13]

Tetracyclines are used so extensively in veterinary medicine, providing broad-spectrum agents acting against a wide range of Gram -positive and -negative bacteria.[14-16] However, several studies have reported that *T. pyogenes* is resistant to tetracyclines[3,4,17-22] and tetracycline resistance is due to the acquisition of new genes, often associated with mobile elements.[14,15] Especially, the *tet*(W) gene is thought to play an important role in tetracycline resistance; while, few studies have examined the distribution of tetracycline resistance genes (TCRs) in *T. pyogenes*.[23]

The main objective of this study was to determine the susceptibility of *T. pyogenes* to tetracycline-based anti-biotics agents and identify TCRs.

Materials and Methods

Bacterial strains. Forty-nine individual *T. pyogenes* strains cattle milk (n = 27) and calf synovial fluid (n = 22) obtained from Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkiye, [24] were investigated. The reference strain ATCC 19411 was used as a positive control for each examination.

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Anti-microbial susceptibility. Resistance profiles of 49 *T. pyogenes* strains were determined on Mueller–Hinton agar (Oxoid, Basingstoke, UK) supplemented with 5.00% sheep blood using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocols.[25] The following three tetracyclines (Oxoid) being commonly used in veterinary medicine were selected: Doxcyciline (30.00 µg), oxytetracycline (30.00 µg) and tetracycline (30.00 µg). Not all anti-microbial agents breakpoints are available for the determination of antibiotic susceptibility by disc diffusion test for *T. pyogenes* in CLSI. [25] Thus, the method used in this study was adapted from ones being described for other fastidious Gram-positive organisms.[25] Then, the results were evaluated as antibiotic susceptible, intermediately susceptible and antibiotic resistant.

Primers. Primer pairs specific for 16 TCRs are listed in Table 1.[26-38]

DNA extraction. DNA extractions from *T. pyogenes* isolates were performed according to the instructions of the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, USA). The DNAs were stored for use as template DNA at – 20.00 ˚C until amplification.

Table 1. Tetracycline resistance specific primers.

Genes	Primer sequence 5'-3'	Amplicon size (bp)
***tet*(A)**	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
***tet*(B)**	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
***tet*(C)**	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
***tet*(D)**	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
tet(E)	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278
tet(G)	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	468
tet(G)	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	844
tet(K)	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169
tet(L)	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267
tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406
tet(O)	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515
tet(S)	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667
tet(P)	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	676
tet(Q)	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	904
tet(X)	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	468
tet(W)	GACAACGAGAACGGACACTATG CGCAATAGCCAGCAATGAACGC	1,843

Polymerase chain reaction (PCR) conditions. Singeleplex PCR assay was carried out for *tet*(W) gene. The protocol was as follows: 25.00 µL reaction volumes containing 3.00 µL MgCl (25.00 mM, Thermo Fisher Scientific Baltics, Vinius, Lithuania), 0.50 µL dNTP (10.00 mM, Thermo Fisher Scientific Baltics), 10.00 pmols of primers and 0.20 µL Taq polymerase (5.00 U μL^{-1}, Thermo Fisher Scientific Baltics) were used and PCR amplifications were performed with the following cycling conditions: 3 min at 94.00 ˚C, followed by 30 cycles of 1 min at 94.00 ˚C (denaturation) and 1 min at 54.00 ˚C (primer annealing), 1 min at 72.00 ˚C (extension) and 7 min at 72.00 ˚C (final extension). Multiplex PCR was performed for Tcrs and these genes were grouped as follows: Group I: *tet*(B), *tet*(C) and *tet*(D), Group II: *tet*(A), tet(E) and tet(G), Group III: *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S) and Group IV: *tet*A(P), *tet*(Q) and *tet*(X) as described by Ng *et al.* Each multiplexed group's PCR reaction mix concentration and amplification conditions were carried out following the previous report.[39]

Results

Antibiotic resistance. As shown in Table 2, 3/49 (6.12%) of the isolates were susceptible to all tetracyclines. Moreover, 43 (87.80%) and 21 (42.86%) of the isolates were resistant to oxytetracycline and tetracycline, respectively, and three isolates were resistant to doxycycline (6.12%). In addition, 7/32 (2.00%) and 9/32 (1.00%) of the isolates were resistant to two and three types of tetracyclines, respectively. Of the strains, 6/32 (18.80%) were resistant to all three tested anti-microbial agents (Fig. 1).

Table 2. Anti-microbial susceptibility results of 49 *Trueperella pyogenes* isolates. Data are presented as number of isolates (%).

Anti-microbials	Sensitive	Intermediate	Resistant
Doxycycline	43 (87.80)	3 (6.12)	3 (6.12)
Tetracycline	8 (16.32)	20 (40.81)	21 (42.86)
Oxytetracycline	3 (6.12)	3 (6.12)	43 (87.80)

Antibiotic resistance genes distribution. Of the 49 *T. pyogenes* isolates, 21 (42.85%) carrying TCRs were identified; 12 (24.49%) with *tet*(A) and 9 (18.37%) with *tet*(W) are presented in Figure 2. In addition, 2 (4.08%) were found to carry both *tet*(A) and *tet*(W) genes. The *tet*(A) gene was identified in 11 (91.75%) isolates resistant to doxycycline, 9 (75.00%) isolates resistant to tetracycline and 1 (8.33%) isolate resistant to oxytetracycline. Moreover, 9 (100%) of the isolates resistant to oxytetracycline and 3 (33.33%) of the isolates resistant to tetracycline were found to possess the *tet*(W) gene. Both *tet*(A) and *tet*(W) genes exhibited 100% resistance against tetracycline and oxytetracycline; but none were resistant to doxycycline (Table 3).

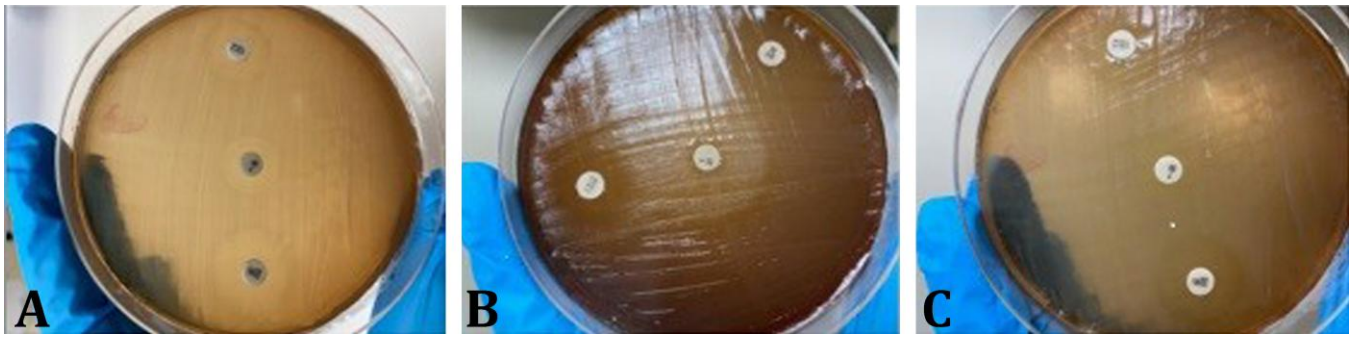

Fig. 1. Kirby-Bauer disc diffusion susceptibility test on *Trueperella pyogenes* Mueller-Hinton agar supplemented with 5.00% sheep blood with **A)** doxycycline (30.00 µg), **B)** tetracycline (30.00 µg) and **C)** oxytetracycline (30.00 µg).

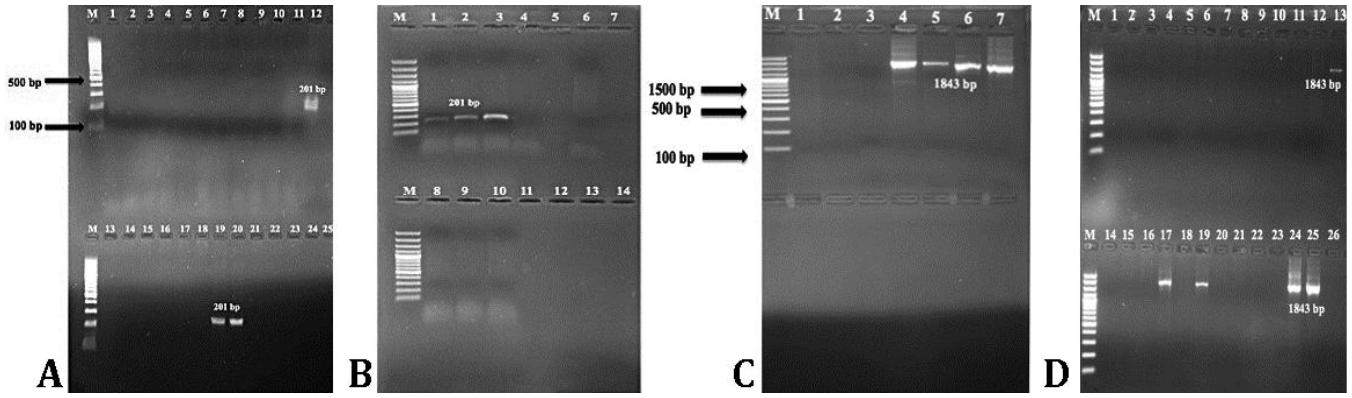

Fig. 2. Multiplex (A and B) and singleplex (C and D) PCR assay being performed using group II (*tet*(A), *tet*(E) and *tet*(G)) and *tet*(G) primers. **A)** M: 100 bp marker; Lanes 12, 19 and 20: *tet*(A) positive PCR products; Lanes 1-11,13-18 and 21-24: Negative; Lane 25: Negative control. **B)** M: 100 bp marker; Lanes 1-3: *tet*(A) positive PCR products; Lanes 4-6 and 8-10: Negative; Lane 7: Negative control. **C)** M: 100 bp marker; Lanes 4-7: *tet*(W) positive PCR products; Lanes 2 and 3: Negative; Lane 1: Negative control. **D)** M: 100 bp marker; Lanes 13, 17, 19, 24 and 25: *tet*(W) positive PCR products; Lanes 1-12,14-16,18 and 20-23: Negative; Lane 24: Negative control.

Table 3. Correlations between *tet*(A) and *tet*(W) genes with tetracyclines agents. Data are presented as number of isolates (%).

Anti-microbials	*tet* A			*tet* W			*tet* both		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Doxycycline	10 (83.33)	1 (8.33)	1 (8.33)	9 (100)	-	-	2 (100)	-	-
Tetracycline	1 (8.33)	2 (16.70)	9 (75.00)	-	6 (66.70)	3 (33.33)	-	-	2 (100)
Oxytetracycline	1 (8.33)	-	11 (91.70)	-	-	9 (100)	-	-	2 (100)

Discussion

Tetracyclines, including tetracycline, doxycycline, chlortetracycline, oxytetracycline and minocycline are commonly used for the treatment of bacterial infections in livestock animals.[40-42] It is well accepted that tetracycline resistance causes resistance to other antibiotics.[43] The purpose of this study was to investigate phenotypic and genotypic tetracycline resistance among the 49 *T. pyogenes* isolates. The highest anti-microbial resistance patterns were observed in 43 (87.80%) isolates, and 21 (42.86%) of the isolates were resistant to oxytetracycline and tetracycline. In addition, 87.80% were susceptible to doxycyciline, being excepted based on previous findings.[44] The high levels of tetracycline[3,4,17,20,45] and oxytetracycline resistance[18,46,47] were similar with the previous studies.[23] In contrast, Ribeiro *et al*., have reported *T. pyogenes* isolates being sensitive to tetracycline.[5]

In this study, singleplex and multiplex PCR assays were used to detect TCRs in *T. pyogenes* isolates. To the best of our knowledge, this is the first report examining the prevalence of these genes in *T. pyogenes* isolates in Turkiye. Here, we used four multiplex PCR groups to detect 15 TCRs (*tet*(A), (B), (C), (D), (E), (G), (H), (K), (L), (M), (O), (P), (Q), (S) and (X)) and singleplex PCR to detect the *tet* (W) gene. It is thought to reveal the screening of the *tet*(A) and *tet*(W) genes in this study which are associated with efflux and/or ribosomal protection mechanisms. This is the first report to identify the *tet*(A) gene in *T.* pyogenes isolates. Although the *tet*(A) gene has also been reported to be a common *tet* resistance gene in Gram-negative genera[48], it is notable that, in this study, the *tet*(A) genes were found among the Gram-positive bacteria. Not suprisingly, the *tet*(W) gene is the fourth most common TCR based on previous studies.[1,3,25,48,49] The predominant hypothesis for the presence of the *tet*(W) in commensal

bacteria, such as *T. pyogenes,* is thought to be due to the acquisition from the ruminal wall of animals.[1]

As mentioned above, the *tet*(A) gene was found in 11(91.75%), 9(75.00%) and 1(8.33%) isolates being resistant to doxycycline, tetracycline and oxytetracycline, respectively. Also, 9 (100%) and 3 (33.33%) isolates being resistant to oxytetracycline and tetracycline possessed the *tet*(W) gene, respectively. In this study, the prevalence of tetracycline resistance due to the *tet*(A) gene was greater than the *tet*(W) gene, which is in contrast with findings from previous studies.[1,23,49] According to Zhang *et al*., the *tet*(W) gene may confer antibiotic resistance, particularly multi-drug resistance; but, since three tetracycline preparations were used in this study, these findings were not confirmed.[23] The presence of *tet*(A) and *tet*(W) genes exhibited 100% resistance against tetracycline and oxytetracycline; but, not doxycycline. Further, the lack of resistance genes in some resistant samples could have been due to the indefinite phenotypic resistance, lack of gene expression or other resistance mechanisms.[43]

The present study provides an overview of susceptibility to commonly used tetracycline preparations and the distribution of TCRs among *T. pyogenes* isolates. The findings in this study are important, since tetracycline resistance remains a significant concern and will contribute to the selection of effective anti-microbial agents against this pathogen in veterinary medicine. This is the first report of the *tet*(A) gene being present in *T. pyogenes*; while, the detection of *tet*(W) confirms its common presence in various bacterial isolates as reported previously. In addition to treatment, control measures should be chosen and improved depending on the type of *T. pyogenes* infection.

Acknowledgments

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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