# Virulence Genes, Biofilm Production and Antibiotic Susceptibility in *Trueperella pyogenes* Isolated from Cattle

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## ABSTRACT

*Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) is a commensal bacterium present on the upper respiratory and urogenital tract mucosa of domestic and wild animals. It is also the most common opportunistic pathogen and isolated from the infections of cattle such as mastitis, metritis, arthritis and pneumonia. The aim of this study was to determine virulence genes (*plo*, *nanH, nanP, cbpA, fimA, fimC, fimE, fimG*) by polymerase chain reaction (PCR), biofilm production by modified microplate test and antibiotic susceptibilities by disc diffusion methods in 44 *T. pyogenes* isolated from various samples of cattle. *Plo* and *fimA* genes were detected in all isolates. *CbpA, nanH,* and *nanP* genes were determined in 6.8%, 61.3%, 84.1% of the isolates, respectively. *FimC, fimE* and *fimG* genes were found in 81.8%, 81.8% and 34.1% of the isolates, respectively. While all *T. pyogenes* isolates were found to be susceptible to amoxicillin-clavulanic acid, 97.7% were susceptible to cefoperazone, 95.45% to amoxicillin, ampicillin, florfenicol and penicillin and 75% to enrofloxacin and cloxacillin. Also all isolates were resistant to neomycin, 84.1% to oxytetracycline, 86.4% to gentamicin and 47.7% erythromycin. The majority of *T. pyogenes* isolates (88.6%) produced biofilms. In conclusion, this study reported that *T. pyogenes* might be a primary pathogen in mastitis, metritis, arthritis, pleuritis and abortion. The majority of *T. pyogenes* isolates had the capability to produce of biofilm that could result in difficulties in the treatment of *T. pyogenes* infections. Also the presence of genes encoded virulence factors may play important roles in the pathogenesis of the infections.

**Keywords:** *T. pyogenes;* Virulence Genes; Biofilm; Antibiotic Susceptibility; Cattle.

## INTRODUCTION

*Trueperella (Arcanobacterium, Actinomyces, Corynebacterium) pyogenes* is a Gram-positive, β-haemolytic, nonmotile, non-spore forming, rod-shaped bacterium (1), commonly found in mucosal surfaces of the upper respiratory tract, urogenital and gastrointestinal system of domestic animals such as cattle, sheep, goats and pigs (1). It also causes suppurative infections in different animals (2, 3, 4, 5, 6, 7). *T. pyogenes* causes serious economic losses in livestock and thus is considered an important opportunistic pathogen in veterinary medicine. Although *T. pyogenes* is generally viewed as the primary pathogen in *T. pyogenes* infections, it is isolated from mixed infections as well. Antibiotics are often used in treatment of infections such as metritis, mastitis, pneumonia, and arthritis in cattle (8, 9, 10). Determining the susceptibility of *T. pyogenes* to antibiotics will help veterinary personnel with antibiotic selection and the treatment of *T. pyogenes* infections (5, 6, 11, 12, 13). Although *T. pyogenes* has been shown to be susceptible to different antibiotics using *in vitro* antibiotic susceptibility tests, treatment with these antibiotics is generally unsuccessful. This condition may be associated with biofilm production of *T. pyogenes,* or use of the antibiotics for an extend period of time (5, 8).

A number of virulence factors contribute to the pathogenicity of *T. pyogenes*, such as pyolysin (*plo*), neuraminidase (*nanH*, *nanP*), and collagen-binding protein (*cbpA*) (14, 15). Pyolysin (*plo*) promotes haemolysis and lysis of immune system cells, and a most important virulence factor. It was found

in all *T. pyogenes* isolates (1, 2). Collagen-binding protein is required for adhesion of *T. pyogenes* to collagen-rich tissue (16). Two neuraminidase enzymes are important virulence factors necessary for *T. pyogenes* to adhere to host epithelial cells, and these enzymes reduce mucus viscosity and decrease the half-life of secretory immunoglobulin A (*sIgA*) (1). *T. pyogenes* has also expressed surface exposed proteins such as fimbriae, which is required for adhesion and colonisation to host tissue encoded by the *fimA, fimC, fimE* and *fimG* genes (17).

The aim of the present study was to identify virulence factor genes, biofilm production and antibiotic susceptibility of *T. pyogenes* isolated from various cattle samples and to explain the role of virulence factors in *T. pyogenes* infections.

## MATERIAL AND METHODS

### Bacterial isolation and identification

This study was made in the Burdur province, located in between the Aegean, Middle Anatolia and Mediterranean, in southwest of Turkey. This region is 7,135 $km^2$ and the maximum altitude is 1000 m above sea level. The climate of Burdur city is almost maritime semiarid. In this study, fourty-four *T. pyogenes* isolates were obtained from dairy cattle (milk (n=27), vaginal fluid (n=6), abscesses (n=3), pleural fluid (n=1), aborted fetus (n=1) and calf samples (synovial fluid, n=6) brought to Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology.

The samples were cultured on Blood agar (Oxoid Ltd., Hampshire, UK) supplemented with 5% defibrinated sheep blood for 48 hours at 37°C in 5% $CO_2$. *T. pyogenes* colonies were identified by conventional bacteriological methods (Gram staining, colony morphology, haemolysis, catalase test, oxidase test, CAMP test (18). The isolates were stored at -80°C until use.

### DNA isolation

*T. pyogenes* isolates and the reference strain (ATCC 19411) were cultured on blood agar supplemented with 5% sheep blood for 48 h, at 37°C in 5% $CO_2$. For each isolates, $2x10^9$ bacterial cells were harvested in microcentrifuge tube by centrifugation for 10 minutes at 5000xg. Supernatant was discarded and the pellet was resuspend in 180 µl of Gram positive bacteria lysis buffer added lysozyme and DNA extraction was performed according to the manufacturer instructions by using Genomic DNA purification kit (Genejet, Thermo Scientific, Vilnius, Lithuania). DNA samples were stored at -20°C until use.

### Screening of genes encoding virulence factors

The presence of eight virulence genes (*plo, nanH, nanP, cbpA, fimA, fimC, fimE* and *fimG*) in *T. pyogenes* were investigated by polymerase chain reaction (PCR). Primers, target gene, PCR product size and references used for PCR are presented in Table 1.

**Table 1:** Primers, target gene, PCR product size used to detect genes encoding *T. pyogenes* virulence factors.

| Target gene | Virulence factor | Primer sequence (5'-3') | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *Plo* | Pyolysin | F-GGC CCG AAT GTC ACC GC<br>R-AAC TCC GCC TCT AGC GC | 270 | Jost *et al.*, 2002 |
| *NanH* | neuraminidase H | F-CGC TAG TGC TGT AGC GTT GTT AAG T<br>R-CCG AGG AGT TTT GAC TGA CTT TGT | 781 | Silva *et al.*, 2008 |
| *NanP* | neuraminidase P | F-TTG AGC GTA CGC AGC TCT TC<br>R-CCA CGA AAT CGG CCT TAT TG | 150 | Silva *et al.*, 2008 |
| *CbpA* | collagen-binding protein | F-GCA GGG TTG GTG AAA GAG TTT ACT<br>R-GCT TGA TAT AAC CTT CAG AAT TTG CA | 124 | Silva *et al.*, 2008 |
| *FimA* | fimbriae A | F-CAC TAC GCT CAC CAT TCA CAA G<br>R-GCT GTA ATC CGC TTT GTC TGT G | 605 | Silva *et al.*, 2008 |
| *FimC* | fimbriae C | F-TGT CGA AGG TGA CGT TCT TCG<br>R-CAA GGT CAC CGA GAC TGC TGG | 843 | Silva *et al.*, 2008 |
| *FimE* | fimbriae E | F-GCC CAG GAC CGA GAG CGA GGG C<br>R-GCC TTC ACA AAT AAC AGC AAC C | 775 | Silva *et al.*, 2008 |
| *FimG* | fimbriae G | F-ACG CTT CAG AAG GTC ACC AGG<br>R-ATC TTG ATC TGC CCC CAT GCG | 929 | Silva *et al.*, 2008 |

PCR reactions performed in 25 µl reaction volume (5 µl DNA sample, 12.5 µl PCR master mix(2X) (Thermo Scientific, Lithuania), 1 µl each primer (100 pmol), 5.5 µl dd$H_2O$) in a Thermal cycler (Apollo, ATC-401, Ramsey, Minnesota, USA). An initial denaturation (94˚C, 3 min) was followed by 35 cycles containing denaturation in 95˚C, 1 min, annealing in 60˚C, 1 min and extension in 72˚C for 3 min) (2, 15). PCR products (10µl) were electrophoresed at 1.5% agarose gel, stained with ethidium bromide (0.5µg/ml). The bands were visualized under the UV light (Edas 290, Eastman Kodak Company, Rochester, NY, USA).

### Biofilm Production

The capacity of biofilm production of *T. pyogenes* was evaluated according to the modified microplate test described by Stepanovic *et al.* (2000). Fourty-four *T. pyogenes* isolates and reference *A. pyogenes* ATCC 19411 strain were cultured in brain heart infusion broth (BHIB, Oxoid Ltd., Hampshire, UK) supplemented 5% foetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) for 24 h at 37˚C in 5% $CO_2$. After 500µl culture was taken and centrifuged 5 min at 10000 rpm. Supernatant was thrawed and the pellet was resuspended in 500 µl BHIB. Subsequently, 180 µl BHIB was added to sterile 96 wells (200 µl/well) of a polystyrene microplate (Corning, Costar 3599, NY). Each inoculum (20 µl) was added to three wells and microplates were incubated for 24 h at 37˚C in 5% $CO_2$. Then, the wells were washed three times with phosphate buffered saline solution (PBS) (pH 7.2) and unattached bacterial cells were removed from wells. Methanol (Merck, KGaA, Darmstadt, Germany) added to wells and microplates were incubated at room temperature. Then microplates were dryed and biofilms were stained crystal violete for 10 minute. Microplates were washed three times with distilated water and dryed 55-60 ˚C. The isolates were dissolved with 33% (v/v) glacial acetic acide (Merck, KGaA, Darmstadt, Germany). Microplates were evaluated at an optical density (OD) 590 nm by ELISA reader (RT-2100C, Rayto and Analytical Sciences Co Ltd., Shenzhen, PRC). *A. pyogenes* 11941 was used to be positive control. The equal volume of sterile medium served as a negative control.

### Antibiotic susceptibility tests

The antibiotic susceptibility of *T. pyogenes* isolates were tested with following antibiotics: amoxicillin (25µg, BHIB, Oxoid Ltd., Hampshire, UK), amoxicillin-clavulanic acid (30µg, BHIB, Oxoid Ltd., Hampshire, UK), ampicillin (25µg, BHIB, Oxoid Ltd., Hampshire, UK), enrofloxacin (5µg, BHIB, Oxoid Ltd., Hampshire, UK), erythromycin (15µg, BHIB, Oxoid Ltd., Hampshire, UK), florfenicol (30µg, BHIB, Oxoid Ltd., Hampshire, UK), gentamicin (10µg, BHIB, Oxoid Ltd., Hampshire, UK), cloxacillin (5µg, BHIB, Oxoid Ltd., Hampshire, UK), lincomycin (10µg, BHIB, Oxoid Ltd., Hampshire, UK), neomycin (30µg, BHIB, Oxoid Ltd., Hampshire, UK), oxytetracyclin (30µg, BHIB, Oxoid Ltd., Hampshire, UK), penicillin G (10U, BHIB, Oxoid Ltd., Hampshire, UK), cefoperazone (75µg, BHIB, Oxoid Ltd., Hampshire, UK), ciprofloxacin (5µg, BHIB, Oxoid Ltd., Hampshire, UK). There is no Clinical and Laboratory Standarts Institute (CLSI) method for the determination of antibiotic susceptibility by disc diffusion test for *T. pyogenes* in CLSI 2013 (20). Thus, the method was adapted from described for other fastidious Gram positive organisms in this study (CLSI 2013). The isolates were incubated in BHIB, for 6-8 h at 37˚C in 5% $CO_2$ and bacterial culture was inoculated onto Mueller Hinton agar supplemented with 5% sheep blood by swab. The antibiotic discs were located on the surface of agar and the plates were incubated for 24 h, at 37˚C, in 5% $CO_2$. Then, the results were evaluated as antibiotic susceptible, intermediately susceptible and antibiotic resistant.

## RESULTS

### Isolation and Identification of *T. pyogenes*

Forty-four *T. pyogenes* bacteria were isolated from clinical samples from cattle. The biochemical characteristics of *T. pyogenes* isolates and the *A. pyogenes* ATCC 19411 reference strain are found similar. All of the *T. pyogenes* isolates and reference strain showed β-haemolysis on blood agar base with 5% sheep blood for 24-48 hours at 37˚C. However, *T. pyogenes* isolates and the reference strain were found positive with *S. aureus* on the CAMP test.

### Determination of virulence genes

In this study, *plo, cbpA, nanH, nanP, fimA, fimC, fimE* and *fimG* genes were detected at different rates in *T. pyogenes* isolates (Table 2). The *Plo* gene was detected in all *T. pyogenes* isolates. Only three isolates carried the *cbpA* gene. The *NanH* and *nanP* genes, which encode neuraminidase were found in 27

**Table 2:** The virulence genes of *T. pyogenes* strains (44 isolates)

| Samples (number of isolates) | Virulence genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Plo* | *nanH* | *nanP* | *cbpA* | *fimA* | *fimC* | *fimE* | *fimG* |
| Milk (n=27) | 27 | 17 | 22 | 2 | 27 | 23 | 23 | 9 |
| Vaginal fluid (n=6) | 6 | 6 | 5 | 0 | 6 | 6 | 4 | 1 |
| Synovial fluid (n=6) | 6 | 3 | 5 | 1 | 6 | 5 | 5 | 3 |
| Abscesses (n=3) | 3 | 1 | 3 | 0 | 3 | 2 | 2 | 1 |
| Pleural fluid (n=1) | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| Aborted fetus (n=1) | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| **Total (n=44)** | **44** | **27** | **37** | **3** | **44** | **36** | **36** | **15** |

and 37 of the isolates, respectively. The *NanH* and *nanP* genes occurred together in 21 (47.7%) of the isolates (Table 3). While *fimA* was found in all of the isolates, *fimC, fimE* and *fimG* genes were identified in 36, 36 and 15 of the isolates, respectively (Table 2).

All *T. pyogenes* isolates recovered from cattle with mastitis carried the *plo* and *fimA* genes. The *cbpA*, *nanH* and *nanP* genes in these isolates were found in 2, 17 and 22 of the isolates respectively (Table 2). Two of the isolates did not carry the *nanH* and *nanP* genes. Twenty-seven *T. pyogenes* isolates carried the least 2 fimbrial genes (Table 3). All of the *T. pyogenes* isolated from cattle with metritis carried the *plo*, *nanH*, *fimA* and *fimC* genes, but not the *cbpA* genes. Five isolates carried both the *nanH* and *nanP* genes (Table 3). Six isolates recovered from synovial fluid were positive for the *plo* and *fimA* genes. One isolate did not carry *nanH* and *nanP* genes. The *cbpA* gene was not present in these isolates. Fimbrial genes were found in different rates in these isolates (Table 3). *T. pyogenes* isolates (n=3) isolated from samples of cattle abscesses were positive for *plo*, *nanP* and *fimA* genes, but not *cbpA*. Two isolates recovered from pleural fluid and aborted fetal tissue were positive for *plo*, *nanP*, *fimA* and *fimE* genes (Table 3). In the reference *A. pyogenes* ATCC 19411 strain, *plo*, *nanH*, *nanP* and *fimC* genes were detected, but not *cbpA*, *fimA*, *fimE* and *fimG*.

## Biofilm production and antibiotic susceptibility test results

Biofilm production was identified in 39 (88.6%) of the *T. pyogenes* isolates and in the *A. pyogenes* ATCC 19411 reference strain. The antibiotic susceptibility test was performed on 44 isolates and on *A. pyogenes* ATCC 19411. Susceptibility of the isolates to 14 antibiotics which are commonly used in veterinary medicine was determined by using the disc diffusion test. The isolates were 100% susceptible to amoxicillin clavulanic acid, 97.7% to cefoperazone, 95.45% to amoxicillin, ampicillin, florfenicol and penicillin, and 75% to enrofloxacin and cloxacillin. All *T. pyogenes* isolates were 100% resistant to neomycin, 84.1% to oxytetracycline and 86.4% to gentamicin (Table 4). The antibiotic susceptibilities of the isolates were not showed differences according to the samples. *T. pyogenes* isolates were found resistant to least 3 antibiotics in this study. *A. pyogenes* ATCC 19411 was susceptible to the 14 antibiotics.

**Table 3:** Genotypes of *T. pyogenes* isolates from cattle samples (44 isolates)

| Samples | Genotypes | Isolates | |
|---|---|---|---|
| | | Number | % |
| Milk | *plo, nanH, nanP, fimA, fimC, fimE, fimG* | 3 | 11.1 |
| | *plo, nanH, nanP, cbpA, fimA, fimC, fimE* | 1 | 3.7 |
| | *plo, nanH, nanP, fimA, fimE, fimG* | 2 | 7.4 |
| | *plo, nanH, nanP, fimA, fimC, fimE* | 5 | 18.5 |
| | *plo, nanH, nanP, fimA, fimC, fimG* | 1 | 3.7 |
| | *plo, nanH, fimA, fimC, fimE, fimG* | 1 | 3.7 |
| | *plo, nanP, cbpA, fimA, fimC, fimE* | 1 | 3.7 |
| | *plo, nanH, fimA, fimC, fimE* | 2 | 7.4 |
| | *plo, nanP, fimA, fimC, fimE* | 4 | 14.8 |
| | *plo, nanP, fimA, fimE, fimG* | 2 | 7.4 |
| | *plo, fimA, fimC, fimE* | 2 | 7.4 |
| | *plo, nanH, fimA, fimC* | 1 | 3.7 |
| | *plo, nanP, fimA, fimC* | 2 | 7.4 |
| Uterine secretion | *plo, nanH, nanP, fimA, fimC, fimE, fimG* | 1 | 16.7 |
| | *plo, nanH, nanP, fimA, fimC, fimE* | 2 | 33.3 |
| | *plo, nanH, nanP, fimA, fimC* | 2 | 33.3 |
| | *plo, nanH, fimA, fimC, fimE* | 1 | 16.7 |
| Synovial fluid | *plo, nanH, nanP, cbpA, fimA, fimC, fimE* | 1 | 16.7 |
| | *plo, nanH, nanP, fimA, fimC, fimE* | 1 | 16.7 |
| | *plo, nanP, fimA, fimC, fimE, fimG* | 1 | 16.7 |
| | *plo, fimA, fimC, fimE, fimG* | 1 | 16.7 |
| | *plo, nanH, nanP, fimA, fimC* | 1 | 16.7 |
| | *plo, nanP, fimA, fimE, fimG* | 1 | 16.7 |
| | *plo, nanH, nanP, cbpA, fimA, fimC, fimE* | 1 | 16.7 |
| Abscesses | *plo, nanH, nanP, fimA, fimC, fimE* | 1 | 33.3 |
| | *plo, nanP, fimA, fimE, fimG* | 1 | 33.3 |
| | *plo, nanP, fimA, fimC* | 1 | 33.3 |
| Pleural fluid | *plo, nanP, fimA, fimE* | 1 | 100 |
| Aborted fetus | *plo, nanP, fimA, fimE, fimG* | 1 | 100 |

**Table 4:** *In vitro* susceptibility to 14 antibiotics of *T. pyogenes* isolates

| Antibiotic | Susceptible | | Resistant | |
|---|---|---|---|---|
| | n | % | N | % |
| Amoxicillin-clavulonic acid | 44 | 100 | 0 | 0 |
| Amoxicillin | 42 | 95.5 | 2 | 4.5 |
| Ampicillin | 42 | 95.5 | 2 | 4.5 |
| Cefoperazon | 43 | 97.7 | 1 | 2.3 |
| Ciprofloxacin | 31 | 70.5 | 13 | 29.5 |
| Cloxacillin | 33 | 75 | 11 | 25 |
| Enrofloxacin | 33 | 75 | 11 | 25 |
| Erythromycin | 23 | 52.3 | 21 | 47.7 |
| Florfenicol | 42 | 95.5 | 2 | 4.5 |
| Gentamicin | 6 | 13.6 | 38 | 86.4 |
| Lincomycin | 25 | 56.8 | 19 | 31.8 |
| Neomycin | 0 | 0 | 44 | 100 |
| Oxytetracycline | 7 | 15.9 | 37 | 84.1 |
| Penicillin G | 42 | 95.5 | 2 | 4.5 |

## DISCUSSION

*T. pyogenes* is an important opportunistic pathogen causing suppurative lesions in livestock animals such as cattle, sheep and pigs (2, 3, 4, 6). It is also present on mucosal surfaces of the upper respiratory tract, urogenital and gastrointestinal system of animals (1, 3, 15, 21). This study examines the biochemical properties, virulence factor genes, biofilm production and antibiotic susceptibility of 44 *T. pyogenes* isolates from cattle. All *T. pyogenes* isolates and the reference strain displayed β-haemolysis on blood agar with 5% sheep blood and a strong positive reaction on the synergistic CAMP test with *S. aureus* producing β-haemolysis. These results have been reported by several other researchers as well (23, 24, 25).

*T. pyogenes* has a number of virulence factors that contribute to its pathogenicity. In this study, as expected, the *plo* genes were present in all *T. pyogenes* isolates and in the *A. pyogenes* ATCC 19411 reference strain. Pyolysin (*plo*) causes lysis in immune cells, murine macrophages and red blood cells in various animals. Therefore, pyolysin is the most virulence factor (14). Researchers from different parts of the world have reported that all *T. pyogenes* strains carry the *plo* gene (2, 3, 4, 5, 7, 25). The *cbpA* gene, which encodes a collagen-binding protein, plays a role in adhesion to collagen-rich host cells (1). In the present study, it was determined that 6.9 % of isolates (mastitis=2, synovial fluid=1) carried the *cbpA* gene. The *cbpA* gene was reported in 21% of *T. pyogenes* isolated from mastitis by Zastempowska and Lassa (2012). Hadimli and Kav (2011) determined that the *cbpA* gene found in 58.8% of *T. pyogenes* isolates originated from different samples of sheep and cattle. Rzewuska *et al.* (2012) detected the *cbpA* gene in *T. pyogenes* isolated from abscesses in the spleen, lymph nodes, lungs and purulent uterine secretions in European bison (25). Santos *et al.* (2010) reported that 1.4% of *T. pyogenes* isolates were recovered from uterine samples. However, Silva *et al.* (2008) reported the presence of the *cbpA* gene in all *T. pyogenes* isolates recovered from the uterus (9). In the present study, the *cbpA* gene could not be detected in cattle samples (abscesses and aborted fetal tissue as well as pleural and uterine secretions). Although mammary and uterus tissue are collagen-rich, in this study, only two *T. pyogenes* isolates recovered from mastitis tissue carried the *cbpA* gene. In this study, we determined that *T. pyogenes* isolates carried the *nanH* and *nanP* genes at different rates and that the *nanP* gene was present at higher rates than the *nanH* gene in the isolates. Similarly, some researchers have reported that the *nanP* gene was detected at a higher rate than the *nanH* gene, too (4, 25). We considered that the differences in the rates of genes encoding neuraminidase may be host specific.

The major fimbrial subunit is encoded by *fimA*, *fimC*, *fimE* and *fimG* genes. The presence of these genes has been reported at different rates in *T. pyogenes* isolates (2, 3, 5, 7, 23). In the present study, f*imA* was detected in all isolates, while *fimC*, *fimE*, *fimG* were found in 81.8%, 79.55% and 29.5% of isolates respectively. Similarly, researchers have stated that *fimA* is found in all *T. pyogenes* isolates (2, 5, 25). Zastempowska and Lassa (2012) reported that *fimA* was found in all of the isolates recovered from mastitis and *fimC, fimE* and *fimG* genes were identified in 88%, 91% and 18% of isolates respectively. But, Hadimli and Kav (2011) reported that *fimA* was present in 96% of isolates originating from sheep and dairy cows. Also, Santos *et al.* (2010) detected the presence of *fimA* in 76.4% of *T. pyogenes* isolated from the vaginal fluid of cattle. In the present study, *fimC* and *fimE* genes were detected at a high rate in *T. pyogenes* isolates. Our results are similar to the findings of Silva *et al.* (2008), Hadimli and Kav (2011), Rzewuska *et al.* (2012), and Zhao *et al.* (2013). But, Al Tarazi *et al.* (2012) reported that the *fimA* gene was determined in only one of two *T. pyogenes* isolates originating from camels, and these two isolates carried the *fimE* genes, but no *fimC* genes (23). In the present study, there

is less *fimG* than other virulence genes, which is similar to the findings of other researchers (4, 5, 25).

In the present study, biofilm production was identified in 88.6% of *T. pyogenes* isolates. All of the isolates recovered from milk with mastitis were positive for biofilm production. Zhao *et al.* (2011) reported that biofilm production was positive in 94.4% of *T. pyogenes* isolated from deer abscesses. The treatment of *T. pyogenes* mastitis is generally difficult and results unsuccessful, which may be due to the biofilm production of *T. pyogenes* isolates. Biofilm production plays an important role in pathogenesis and makes it difficult to treat persistent infections. The bacteria in biofilm are less exposed to the host's immune response and antibiotics (26). Antibiotics are generally used to treat *T. pyogenes* infections. The susceptibility of *T. pyogenes* to antibiotics has been shown in various studies (3, 7, 12, 13). Identifying antibiotic susceptibility of *T. pyogenes* isolates will help veterinary personnel in the selection of antibiotics to treat the infections. In this study, 100% of the isolates were found susceptible to amoxicillin-clavulanic acid. Susceptibility to amoxycillin, ampicillin, cefoperazone, cloxacillin, enrofloxacin, florfenicol and penicillin varied in between 75%-97.7%. It is not surprising, therefore, that some farmers reported treatment failure even though these antibiotics were administered to the animals. The cause of this failure in the treatment of *T. pyogenes* infections is thought to be the fact that *T. pyogenes* isolates may produce biofilm. Because the majority of the strains are able to produce biofilm, this may make treatment of the infections caused by *T. pyogenes* strains difficult even though they are sensitive to antibiotics *in vitro* (13).

In conclusion, we found that the majority *of T. pyogenes* isolates were capable of biofilm production, and other virulence factor genes besides *plo* and *fimA* were found in different rates in the isolates.

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