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Original Article

Detecting Virulence Factors and Antibiotic Resistance Pattern of *Trueperella Pyogenes* Isolated from Bovine Mastitic Milk

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Backgrounds and Aims: Mastitis is a mammalian disease which is considered important due to its potential economic damages. *Trueperella pyogenes*is is one of the important opportunistic pathogens of the mammary glands of cattle. This bacterium can produce acute mastitis infection in dairy cattle. In fact, this bacterium has several virulence genes which contribute to its pathogenicity. Therefore, this study aimed to determine the frequency of this bacterium in the sick cattle in addition to identifying the effective virulence gene in this disease and finally investigating the antibiotic resistance of *T. pyogenes*.

Materials and Methods: In total, 126 milk samples from mastitic cows, were collected and immediately transferred to the laboratory. Isolation and identification of *T. pyogenes* strains were performed using bacteriological methods. Moreover, antimicrobial susceptibility testing was performed using the disk diffusion technique polymerase chain reaction was used to detect *plo, fimA, fimC, cbpA, nanH,* and *nanP* virulence genes.

Results: *T. pyogenes* was detected in 46 milk samples. The expression of *plo* and *fimA* virulence genes was observed in all samples while fimC expression was detected in 84.7% of cases. The most resistance was observed against tetracycline (97.8%) and gentamicin (86.9%), whereasthe least resistance was reported for chloramphenicol (4.3%) and nitrofurantoin (10.9%).

Conclusions: *T. pyogenes* has been regarded as one of the bacteria causing mastitis disease in the cattle. *Plo, fimA,* and *fimC* virulence genes seem to be the main virulence factors of this bacterium.

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Introduction

Trueperella pyogenes which was previously classified in genus *Arcanobacterium* is a non-motile gram-positive anaerobic bacterium [1]. It has been found in urinary tract, gastrointestinal tract, and upper respiratory tract of mammals such as cattle, goat, horse, musk deer, pig, and sheep which is able to cause abscess, mastitis, metritis, and pneumonia. In interaction with other bacteria, this bacterium produces purulent lesions and abscesses in various organs and tissues, entering the blood flow [2, 3].

T. pyogenes has several well-recognized virulence factors which confer its pathogenic potentials. These virulence factors include a hemolysin known as pyolysin (Plo) which promotes the hemolytic activities towards red blood cells and cytolytic activities towards immune cells. Two neuraminidases (NanH and NanP) which are necessary for adhesion to epithelial cells, a collagen-binding protein (CbpA) which is required for attachment to collagen- and fimbriae-rich environments and probably adhesion [3]. This bacterium can produce acute infectious mastitis in the dairy cattle. Mastitis occurs in all female mammals, but it is economically important in dairy cattle due the damages to the milk and dairy industry. The economic loss caused by this disease is due to not only livestock fatality but also the reduction of milk production by the animal and disorders in milk processing which are actually the major detriments [4, 5]. The effectiveness of antimicrobial agents seems not to be sufficient for curing the intramammary infections in this disease. However, it was demonstrated that long-term intensive systemic treatment of primary cases with penicillin has been effective. Furthermore, the use of antibiotic dry cow therapy has been demonstrated to have beneficial effects on reducing the incidence of the disease [6].

Mainly, treatment of diseases caused by this bacterium often requires antimicrobial therapy; however, antibiotic resistant strains of bacteria cause more severe diseases for longer periods of time than their antibiotic-susceptible counterparts. Today, antibiotic resistant in *T. pyogenes* is getting increased. Therefore, identification of resistance pattern of bacteria seems to be so essential in reduction of treatment costs. No previous data has been reported regarding detection of virulence genes, and antimicrobial resistance of *T. pyogenes* strains isolated from cow in Iran. Therefore, this present study was carried out for molecular characterization of *T. pyogenes* strains isolated from bovine mastitic milk.

Materials and Methods

Smplaing and detecting *Trueperella pyogenes*

Overall, 126 bovine mastitic raw milk samples were collected from centers from several geographic regions of Iran, during the summer of 2014. The animals selected for this study were clinically healthy, and the milk samples showed normal physical characteristics. In this study, mastitic milks were identified by the California Mastitis Test (CMT). The study samples (5 mL, in sterile glass containers)

were transported to the laboratory at 4°C within a maximum of 6–12 h after sampling.

The samples were diluted in sterile 0.9% saline and cultivated on blood agar medium (Merck, Germany) at 37°C for 2 days. Assumed isolated *T. pyogenes* strains were cultivated on blood agar medium at 38°C with 5% CO2 for 2 days [7]. Pure cultures of isolates were obtained and genomic DNA was extracted via MiniBEST bacterial genomic DNA extraction kit, version 2.0 (TaKaRa). *16SrRNA* gene was amplified using universal primers UNF (5'-GAGTTTGATCCTGGCTCAG-3') and UNR (5'-GGACTACCAGGGTATCTAAT-3') [6] in 35 cycles consisting of 45 s denaturation at 94°C, 1 min. annealing at 49°C and 1 min. extension at 72°C, followed by a final extension of 72°C for 10 min. Polymerase chain reaction products were visualized using ethidium bromide (0.5 mg/ml) after electrophoresis on 1.0% agarose gels, which was examined under ultraviolet illumination (Uvitec, UK). Fragments were sequenced in an ABI 3730 automated sequencer (Applied Biosystems). Database searches were performed using BLASTN algorithm against Gen bank sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pyolysin-encoding gene (plo) was amplified using specific primers (Table 1) for 30 cycles consisting of 45 s at 94°C, 45 s at 55°C and 1 min. at 72°C, with a final extension step of 72°C for 10 min. As a matter of fact, the amplified PCR products were sequenced and analysed as described above. In all PCR reactions, a DNA thermocycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used.

Detection of genes encoding virulence factors

The genes encoding seven known and putative virulence factors of *T. pyogenes* presented in table 1 were detected through PCR. Primers and reaction conditions were previously described by Silva et al. (2008) and implemented here with slight modifications [8]. All amplifications were carried out in a 25 ml reaction mixture containing 10 pmol of each primer (Genomed, Poland), 0.2 mM of each dNTPs (Fermentas, Lithuania), 2 mM of MgCl2 (Fermentas, Lithuania), 1X Taq polymerase buffer (Fermentas, Lithuania), 1.5 U of Taq DNA polymerase (Fermentas, Lithuania) and 40 ng of genomic DNA. PCR thermal profiles for each amplified gene differed in the annealing temperature which is as follows: a 3-min initial denaturation at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min. annealing at a temperature as shown in table 1, 3 min. extension at 72°C, and a final extension at 72°C for 7 min. The products were separated through 1.7% agarose gel electrophoresis in Tris/Borate/EDTA buffer, stained with ethidium bromide, visualized using a Uvitec Imaging System (UK). In order to confirm the PCR results, a sequencing method was used. Hence, PCR products of some positive samples were purified via a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's recommendations. Single DNA strands were sequenced with an ABI 3730 XL device and Sanger sequencing method (Macrogen, Seoul, South Korea). The sequence of each gene was aligned with the gene sequences recorded in the GenBank database on the NCBI.

Table 1. Primers used for virulence factors detection in *T. pyogenes*

Virulence factor	Target gene	Primer sequence (5'-3')	Annealing temperature
Pyolysin	*Plo*	F-TCATCAACAATCCCACGAAGAG R-TTGCCTCCAGTTGACGCTTT	60
Fimbriae A	*fimA*	F-CACTACGCTCACCATTCACAAG R-GCTGTAATCCGCTTTGTCTGTG	57
Fimbriae C	*fimC*	F-TGTCGAAGGTGACGTTCTTCG R-CAAGGTCACCGAGACTGCTGG	60
Fimbriae G	*fimG*	F-ACGCTTCAGAAGGTCACCAGG R-ATCTTGATCTGCCCCCATGCG	57
Collagen-binding protein	*cbpA*	F-GCAGGGTTGGTGAAAGAGTTTACT R-CTTGATATAACCTTCAGAATTTGCA	60
Neuraminidase H	*nanH*	F-CGCTAGTGCTGTAGCGTTGTTAAGT R-CCGAGGAGTTTTGACTGACTTTGT	60
Neuraminidase P	*nanP*	F-TTGAGCGTACGCAGCTCTTC R-CCACGAAATCGGCCTTATTG	60

Susceptibility testing

12 antimicrobial agents were purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China): penicillin (10 u/disk), tetracycline (30 μg/disk), streptomycin (10 μg/disk), chloramphenicol (30 μg/disk), sulfamethoxazole (25 μg/disk), gentamicin (10 μg/disk), enrofloxacin (5 μg/disk), cephalothin (30 μg/disk), ciprofloxacin (5 μg/disk), trimethoprim (5 μg/disk), and nitrofurantoin (300 μg/disk). Antimicrobial susceptibility tests were carried out for the *T. pyogenes* strains with a final inoculum of 4×10^5 cfu/ml on Müller–Hinton agar medium (HiMedia Laboratories, Mumbai, India, MV1084) supplemented with 5% fetal calf serum at 37°C subjected to 5% CO2 for 24 hours, as described by the Clinical and Laboratory Standards Institute [9]. In addition, *Escherichia coli* ATCC 25922 was used for the quality-control. Medical Ethics Committee of Islamic Azad University of Shahrekord branch approved the study.

Results

Detection and isolation of bacteria

The cultivated bacteria from cattle suffering from mastitis in the mentioned medium were subjected to molecular analysis of *16srRNA* and pyolisin (*plo*) gene. The blast results of the sequenced fragments showed that only 46 milk samples from all 126 contained *T. pyogenes*.

Genes encoding virulence factors

The results obtained from the virulence genes, pyolysin (*plo*), neuraminidase H and P (*nanH*, *nanP*), collagen-binding protein (*cbpA*), and fimbrial subunits genes (*fimA, fimC, fimG*) showed that all 46 isolated *T. pyogenes* in this study contained *plo* and *fimA* virulence genes. In addition, 15.2% (7 cases) and 21.7% (10 cases) of isolates had *nanH* and *nanP* genes, respectively (Table 2).

Table 2. Distribution of virulence gene in *T. pyogenes* strains isolated from bovine mastitic milk

Gene	*Plo*	*fimA*	*fimC*	*fimG*	*nanP*	*cbpA*	*nanH*
Count	46	46	39	12	10	8	7
Percent	100%	100%	84.7%	26%	21.7%	17.3%	15.2%

Antibiotic resistance pattern

T. pyogenes resistance to different antibiotics was examined in the present study. The study results suggested that the most resistance was observed to tetracycline (97.8% of isolated strains), gentamicin (86.9%), and streptomycin (84.8%). Furthermore, only 2 (4.3%) out of all 46 samples were resistant to chloramphenicol. In addition a limited number of bacteria were resistant to nitrofurantoin, cephalexin, and ciprofloxacin. The ultimate results have been demonstrated in table 3.

Table 3. Antibiotic resistance pattern of *T. pyogenes* strains isolated from bovine mastitic milk

Antibiotic	Count	Percent
tetracycline	45	97.8
gentamicin	40	86.9
streptomycin	39	84.8
penicillin	32	69.6
erythromycin	29	63
trimethoprim/ sulphamethoxazole	18	39.1
trimethoprim	12	26.1
enrofloxacin	11	23.9
ciprofloxacin	9	19.6
cephalexin	8	17.4
nitrofurantoin	5	10.9
chloramphenicol	2	4.3

Discussion

Mastitis occurs as a result of acute or chronic infections. Mastitis in livestock is determined through physicochemical as well as microbial changes which appear in the mammary glands. There are a variety of bacteria involved in this disease such as *Trueperella pyogenes*, previously known as *Arcanobacterium pyogenes* [10, 11]. In the present study, *T. pyogenes* was selected as the target bacterium and 126 milk samples from cattle suffering from mastitis were analyzed for the presence of this bacterium. The results from bacterial cultures and *16srRNA* analysis together with *plo* gene sequencing demonstrated that 46 samples contained the bacterium. *T. pyogenes* is a member of a small group of gram positive bacteria capable of producing fimbrea. *FimA* protein coding gene has been widely

distributed in this bacterium. This gene has been detec ted in 94% of isolates from different animals [12]. In addition, it has been present in the uterus of 100% of dairy cattle [8]. The gene responsible for coding *fimA* protein was found in all *T. pyogenes* strains in this study, which was consistent with other studies [13, 14]. Considering the presence of this gene in all examined strains, it is not trivial to assume a role for this gene in pathogenicity of the bacterium.

Previous studies showed that the frequency of genes coding the other fimbrae subunits is different according to the difference between the origins of the bacterium. For example, silva et al. (2008) showed that *fimC* coding gene had a higher frequency in the bacteria isolated from mastitis compared to those isolated from metritis [8]. Furthermore, *fimG* coding gene was more frequent in the isolates from metritis in comparison with mastitis. The present study also demonstrated similar results and the higher frequency of *fimC* compared to *fimG* in *T. pyogenes* strains isolated from dairy cattle suffering from mastitis.

It has been previously demonstrated that the gene coding collagen binding protein (cbpA) was present in 48.9% of *T. pyogenes* bacteria isolated from bovine [15] and in 100% of isolated bacteria from cattle uterus [16]. It should be noted that it was observed in only 17.3% of isolated strains in this study. The difference in the frequency of this gene in *T. pyogenes* strains may be a result of inherent variations between different strains.

T. pyogenes elaborates at least two neuraminidases encoded by *nanH* and *nanP* genes. Studies by Jost et al. (2001, 2002) demonstrated that *nanH* and *nanP* were present in, respectively, 100% and 64.2% of isolated strains from various infections in their experiments [17, 18]. Furthermore, Silva et al. (2008) showed the presence of both *nanH* and *nanP* genes in the bacteria of cattle uterus [8]. The results obtained from investigations by Hijazian et al. (2011) and Zastempowska (2012) on bacteria from cattle inflation were not the same as the previous studies [13, 14]. Similarly, the findings of this study did not reveal the presence of both genes in all bacteria. The frequency of these genes was even much less than that of previous studies. The comparison between the results of this study and those of other studies suggest that these genes are not the main factors in cattle mastitis occurrence.

Penicillin and ampicillin have been widely used for the treatment of infections, however, this approach seems not to be appropriate for dealing with these isolated bacteria [19, 20]. The study results demonstrated that tetracycline, gentamicin, and streptomycin antibiotics were the least effective in isolated *T. pyogenes* growth inhibition in this study; i.e. 80% of these bacteria were resistant to these antibiotics. Moreover, 60% of bacteria were resistant to streptomycin and penicillin antibiotics. According to the low resistance to ciprofloxacin and enrofloxacin, these antibiotics seem to be the best choices for an appropriate treatment approach.

Conclusion

The study results demonstrated the presence as well as effect of *T. pyogenes* in cattle mastitis disease. The *fimC, fimA,* and *plo* genes can be

introduced as the main virulence factors of this bacterium. Furthermore, the antibiotic resistance tests revealed that chloramphenicol and nitrofurantoin can be chosen for mastitis treatment. Chloramphenicol and nitrofurantoin are forbidden antibiotics, and the high antibiotic resistance to them in the present study indicated the irregular and unauthorized uses of these antibiotics in veterinary treatment in Iran. Unfortunately, veterinarians in many fields of veterinary such as large animal internal medicine, poultry, and even aquaculture use these antibiotics considered as a basic one. Therefore, in a very short period of time, antibiotic resistance will appear.

According to findings of the present study, the following items are recommended: (i) using PCR method as an accurate, safe, and fast diagnostic one for accurate detection of pathogens in mastitic milks. (ii) using simple disk diffusion method in order to evaluate the antibiotic resistance of pathogens in mastitis cases; (iii) due to antibiotic resistance especially in *E. coli*, the veterinarians should pay more attention to prescribing the antibiotics; (iv) in order to prevent antibiotic resistance in bacteria, antibiotics should be applied more cautiously in animals, detecting resistance genes, and finally using different antibiotics periodically.

Conflict of Interest

The authors declare no conflicts of interest.

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