Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.040

RESEARCH ARTICLE

Antimicrobial Resistance and Virulence Genes Distribution in *Trueperella pyogenes* Isolated from Dairy Cows with Clinical Mastitis in Liaoning of China

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ARTICLE HISTORY (20-018)

Received: January 18, 2020
Revised: March 27, 2020
Accepted: April 23, 2020
Published online: April 20, 2021

Key words:
Antimicrobial susceptibility
Bovine mastitis
T. pyogenes
Virulence phenotype

ABSTRACT

Trueperella pyogenes is considered as a causative agent of many infections, such as mastitis, endometritis, pneumonia, liver abscessation. *T. pyogenes* can express several virulence genes such as *plo, fimA*, *cbpA*, *nanH* and *nanP* contributing to its pathogenicity. The aim of this study was to provide an investigation about antimicrobial resistance, as well as virulence genes distribution and gene cassettes among *T. pyogenes* isolates from dairy cows with clinical mastitis. The susceptibility to different antimicrobial agents was determined by the Broth Microdilution Method, and virulence genes and gene cassette was detected by polymerase chain reactions (PCRs). There are 10.49% (17/162) of milk samples from dairy cows with mastitis were positive for *T. pyogenes*. High levels of resistance were found to clindamycin (23.53%), oxytetracycline (23.53%), ciprofloxacin (47.06%), sulfamethoxazole/trimethoprim (100%). Moreover, all isolates carried class I integrons, and gene cassette arrays were *aadA9* (2/17) or *aadA5-dfrA17* (3/17). Finally, all isolates harbored *plo nanH* and *fimA* genes, but other genes encoding virulence genes including *fimC*, *fimE*, *nanP* and *cbpA* are ranged from 47.06% to 88.23%. Our study showed *T. pyogenes* isolates from dairy cows with clinical mastitis were susceptible to β-lactams. In addition, all seven virulence genes occurred in isolates, and *plo*, *nanH*, and *fimA* gene showed a significantly higher frequency in *T. pyogenes* of the Liaoning Province, China.

To Cite This Article: Guo Y, Qiu P, Su H, Li Y, Guo Y, Zhang Z, Liu Y, Liu M and Zhang D, 2021. Antimicrobial resistance and virulence genes distribution in *trueperella pyogenes* isolated from dairy cows with clinical mastitis in Liaoning of China. Pak Vet J, 41(3): 329-334. http://dx.doi.org/10.29261/pakvetj/2021.040

INTRODUCTION

Mastitis causes severe economic loss to dairy cows because of therapy costs, losses caused by reduced milk production, discarded milk, increased workload, and culling and replaced dairy cows (Dong *et al*., 2019; Rzewuska *et al*., 2019). Many pathogens are believed to be involved in dairy mastitis, such as *Trueperella pyogenes* (*T. pyogenes*) (Galán-Relaño *et al*., 2020a). *T. pyogenes* is a Gram-positive and rod-shaped bacterium, which was previously named as *Arcanobacterium pyogenes* (Yassin *et al*., 2011). *T. pyogenes* commonly inhabit on the mucous membranes of animals, but it also can lead to various diseases in domestic and wild animals, such as metritis, pneumonia, lymphadenitis, arthritis, otitis, pyodermitis, peritonitis, pyodermitis, endocarditis, umbilical thickening, organ abscesses, and osteomyelitis (Ribeiro *et al*., 2015).

Antimicrobial resistance in bacteria gains a growing concern in human and veterinary medicine. It is essential for monitoring the antimicrobial susceptibility of pathogens because it can provide information for veterinarians to choose the appropriate antibiotic when treating infections (Bengtsson *et al*., 2009). Antibiotic therapy is essential in treating mastitis of dairy cows, but it may lead to antimicrobial resistance, thus monitoring the antimicrobial susceptibility of udder pathogens is essential for long-term efficacy of antibacterial agents. In terms of mechanism of antimicrobial resistance in *T. pyogenes*, a recent study indicated that genomic island type IV secretion system and transposons in genomic isoland in *T. pyogenes* are associated with antimicrobial resistance (Dong *et al*., 2020). However, the antimicrobial susceptibility and mechanism in *T. pyogenes* from dairy

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cows with mastitis are rarely investigated in the Liaoning Province of China.

Several virulence factors contribute to the pathogenicity of *T. pyogenes*. For example, haemolytic extoxin pyolysin (PLO) can lyse red blood cells of animals, and lead to the characteristic β-haemolysis in *T. pyogenes* when growing on blood-containing agar plate. Neuraminidases (NanH and NanP), collagen-binding protein (CbpA) and fimbriae (pili) can promote adhesion to host cells. By expressing these virulence genes, *T. pyogenes* can inhabit on various host tissues and lead to diverse infections (Pietrocola *et al*., 2007).

The aim of this study was to determine the *in vitro* antibiotic sensitivity of *T. pyogenes* isolated from mastitis of dairy cows in the Liaoning Province, northeast part of China, and to identify virulence factor genes among *T. pyogenes* isolates; meanwhile, cassette-integron detection will be crucial for illustrating the mechanism of antibiotic resistance in *T. pyogenes* isolates.

MATERIALS AND METHODS

Herds and animals: In the Liaoning Province of China from June to September 2017, twelve herds were visited, and mean herds size was 75, median 86. A total of 185 milk samples were collected from lactating cows with clinical mastitis. The diagnosis of clinical mastitis was carried out by the trained farm technician according to previous report (Bryan and Taylor, 2009).

Sample collection: Duplicated quarter milk samples were acquired from each cow with clinical mastitis using aseptic technique as previous (Metzqer *et al*., 2018).

***T. pyogenes* isolates:** A 10 μL milk sample from each teat was inoculated onto agar plates containing 5% defibrinated sheep blood and polymyxin B (100U/mL), and the plates were stored in an incubator with 5% CO_2 at 37 ºC for 48 h to isolate *T. pyogenes*. The suspected isolates were confirmed by the API Coryne system (bioMérieux, Shanghai, China). If necessary, the *sodA* was amplified and sequenced to identify the isolate (Hijazin *et al*., 2011).

DNA extraction: A single colony from a fresh bacterial culture from BHI agar was picked and inoculated into 3 mL of sterile BHI containing 8% FBS at 37ºC for 24 h. The DNA extraction was carried out using MiniBEST Bacteria Genomic DNA Extraction Kit (Takara, Dalian, China) as described in the instruction. All DNA preparations were stored at -20ºC until use.

***16S rRNA* gene sequences analysis:** In order to further identify the isolates and analyze their evolution status, *16S rRNA* gene was sequenced. *16S rRNA* gene was amplified using a PCR machine (Bio-Rad, Hercules, CA). Nucleotide sequence was analyzed by DNASTAR software (DNASTAR Inc., Madison, WI) and the program NCBI-BLAST (http://www.ncbi.nlm.nih.gov).

The *16S rRNA* gene of these isolates was analyzed with those from reference strains, including *Arcanobacterium*, *Trueperella* and *Actinobaculum* species. The CLUSTAL W program of MEGA version 5.0 was used to analyze the multiple-sequence alignment.

Antimicrobial susceptibility assay: Antimicrobial resistance was measured by the Broth Micro-dilution Method as described in the Clinical Laboratory Standards Institute Guidelines (CLSI, 2017). For each isolate, inoculums were adjusted to a turbidity equivalent to a 0.5 McFarland standard to carry out antimicrobial susceptibility testing. Trays were kept in an incubator for 24 h at 37ºC and *T. pyogenes* ATCC 19411 was used as a reference strain.

The MIC breakpoints for other veterinary or human pathogens according to CLSI (2017) were selected to interpret MICs as previous (Table 2) (Zastempowska and Lassa, 2012).

Integrase gene and gene cassettes detection: Various types of integon and gene cassettes were detected by PCR as previously (Liu *et al*., 2009). Primers used for the gene cassette region and integrase gene (*intI* I and *intI* II) are listed in Table 1.

Detection of virulence genes: The genes encoding virulence factors were amplified using PCR as reported previously (Zastempowska and Lassa, 2012). Genes including *plo*, *nanP*, *nanH*, *fimA*, *cbpA*, *fimC*, and *fimE* were amplified. Primer sequences are listed in Table 1.

Statistical analysis: It is compared between the presence of virulence genes and signs of clinical mastitis in dairy cows, and mastitis and non-mastitis cases by Chi-Square and Fisher's exact tests.

RESULTS

Prevalence of *T. pyogenes*: In all, 185 udder quarters were sampled in our study. The sample showed duplicated isolates with the same bacterial species within a cow was excluded, and 166 strains from 162 udder quarters in 141 cows were identified. The isolates were as followed: *Staphylococcus aureus* (n=56), *Escherichia coli* (n=40), coagulase-negative staphylococci (n=32), *Streptococcus uberis* (n=14), *Streptococcus dysgalactiae* (n=21), *Streptococcus agalactiae* (n=6) and *T. pyogenes* (n=17).

All 17 isolates recovered from dairy cows with clinical mastitis were cell morphology for *T. pyognees*, and formed small, irregular, white colonies. All *T. pyogenes* isolates exhibited β-haemolysis on MH(A) plates containing blood. Moreover, the suspected isolates were further identified by API coryne strips, and the code for all the isolates was 4212720, which indicated that all the isolates were *Trueperella pyogenes*. Overall, the prevalence of *T. pyogenes* was 9.18% from 185 milk samples from clinical mastitic cows belonging to 5 of 12 dairy herds located in Liaoning of China.

Signs of bovine clinical mastitis were recorded in order to analyze its correlation with virulence genes in *T. pyogenes*. All milk samples from bovines with clinical mastitis possessed clots and swelling within the udder. Frequencies of virulence genes were high, but there was no correlation between signs of bovine clinical mastitis and virulence genes.

Table 1: Primers and PCR conditions used in the study

Primers	Primer sequence (5'→3')	Size of PCR product (bp)	Reference
sodA F	CGAGCTCGCCGACGCTATTGCT	489	Zolg and Philippi-Schulz (1994)
sodA R	GAGCATGAGAATCGGGTAAGTGCCA		
plo F	GGCCCGAATGTCACCGC	270	Jost *et al.* (1999)
plo R	AACTCCGCCTCTAGCGC		
nanH F	GCCCCGAGCACAGCGGAAC	2889	This study
nanH R	TTAGCCCTGACGACGGCGAAT		
nanP F	GGCTCTTGGCGATTCCGTTTG	1296	This study
nanP R	TGGTCGTCCACTCGCTCTTGTC		
cbpA F	GCAGGGTTGGTGAAAGAGTTTACT	124	Silva *et al.* (2008)
cbpA R	GCTTGATATAACCTTCAGAATTTGCA		
fimA F	CACTACGCTCACCATTCACAAG	605	Silva *et al.* (2008)
fimA R	GCTGTAACTCCGCTTTGTCTGTG		
fimC F	TGTCGAAGGTGACGTTCTTCG	843	Silva *et al.* (2008)
fimC R	CAAGGTCACCGAGACTGCTGG		
fimE F	GCCCAGGACCGAGAGCGAGGGC	775	Silva *et al.* (2008)
fimE R	GCCTTCACAAATAACAGCAACC		
16S rRNA F	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	1545	Liu *et al.* (2009)
16S rRNA R	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT		
int I F	CCTCCCGCACGATGATC	280	Liu *et al.* (2009)
int I R	TCCACGCATCGTCAGGC		
int II F	TTATTGCTGGGATTAGGC	233	Liu *et al.* (2009)
int II R	ACGGCTACCCTCTGTTATC		

Table 2: Breakpoints of antimicrobial agents for testing antimicrobial susceptibility against *T. pyogenes* isolates

Antimicrobial agents	Minimal inhibitory concentration breakpoint (μg/mL)		
	Susceptible	Intermediate	Resistant
Amoxicillin$^{\Phi}$	≤0.25		≥2
Ampicillin*	≤0.25		≥0.5
Chlortetracycline†	≤2	4	≥8
Cefotaxime*	≤2	4	≥8
Ceftiofur†	≤2	4	≥8
Clindamycin*	≤0.5	1-2	≥4
Enrofloxacin†	≤0.25	0.5-1	≥2
Erythromycin$^{\blacktriangledown}$	0.25	0.5	≥1
Florfenicol†	≤2	4	≥8
Gentamicin‡	≤4	8	≥16
Oxytetracycline†	≤2	4	≥8
Penicillin$^{\blacklozenge}$	≤0.03		≥0.06
Sulfamethoxazole/Trimethoprim$^{\blacklozenge}$	≤0.12		≥0.25
Tetracycline$^{\Psi}$	≤4		≥8
Tilmicosin†	≤8	16	≥32
Ciprofloxacin	≤1	2	≥4

*MIC breakpoints for Staphylococcus species from CLSI were used. †MIC breakpoints from CLSI for bovine respiratory disease were used. ‡MIC breakpoints from CLSI for undefined pathogens from veterinary. ΦMIC breakpoint was based on the British Society for Antimicrobial Chemotherapy (http://www.bsac.org.uk/_db/_documents/Chapter_3.pdf). ΨMIC breakpoint for tetracycline was based on Streptococcus pneumonia. ▼Breakpoint is according to that for streptococci. ◆Breakpoints for penicillin and sulfamethoxazole/trimethoprim (19/1) are from CLSI (2017) for *Trueperella pyogenes*.

Phylogenetic analysis of *16S rRNA* gene sequences: Our results indicated that these 17 isolates were *T. pyogenes* since all the isolates clustered with *T. pyogenes* ATCC19411 (Fig. 1). It showed that the identity was above 99% to the reference strain by the BLASTN analysis. Isolates showed high similarity with strains belonging to *Trueperella*, with a 97.4% similarity to *Trueperella bernardiae*, 95% to *Trueperella bialowiezensis*, 96.04% to *Trueperella bonasi*. Similar results were observed when isolates in our study was compared with genus *Arcanobacterium*, with 94.74% similarity to *Arcanobacterium hippocoleae*, 94.59% to *Arcanobacterium pluranimaliu*, 94.45% to *Arcanobacterium phocae*, 93.9% to *Arcanobacterium haemolyticum*. The similarities to *Actinobaculum massiliense* and *Actinobaculum urinale* were 91.86% and 93.08%, respectively.

***In vitro* antimicrobial susceptibility test:** All *T. pyogenes* isolates were carried out to antimicrobial susceptibility testing, and antibiotic resistance was observed in ciprofloxacin and oxytetracycline (Table 3 and Fig. 2). Apparently, the *T. pyogenes* isolates was susceptible to β-lactams (including amoxicillin, ampicillin, cefotaxime, cefquinome, ceftiofur, penicillin) and gentamicin, and these isolates showed no resistance against these antimicrobial agents. Similarly, low rate of isolates was resistant to florfenicol (5.88%), tetracycline (5.88%), erythromycin (11.76%), and tilmicosin (11.76%). Conversely, there were higher frequencies of resistance to sulfamethoxazole/trimethoprim (100%), ciprofloxacin (47.06%), oxytetracycline (23.53%), and clindamycin (23.53%).

Results of gene cassettes and the integrase gene: No class II integrons is present in this study, whereas *intI* I was positive among all the isolates (Table 4). Consequently, the sequencing results of gene cassettes indicated that the integrons harbored 1 or 2 gene cassettes including *aadA9* or *aadA5-dfrA17*. Isolates carrying these gene cassettes showed multidrug resistance except for T005 and T015 isolates, these gene cassettes seem to be involved in aminoglycoside resistance (*aadA5* and *aadA9*) and trimethoprim resistance (*dfrA17*).

Genes encoding virulence factors: We detected the distribution of virulence genes among 17 *T. pyogenes* isolates by using PCR method. Our results indicated that *plo*, *fimA* and *nanH* genes were positive among all isolates. Among these isolates, 15 (88.23%) were positive for *fimC* gene, and *fimE* and *nanP* were also showed high occurrences with 82.35% and 70.59%, respectively. The *cbpA* gene was positive in 8 (47.06%) isolates. Among these genotypes, the most detected genotype was *plo fimA fimC fimE nanH nanP*, which was detected in 4 (23.52%) isolates, meanwhile, genotypes including *plo fimA fimC fimE nanH nanP cbpA*, *plo fimA fimC cbpA nanH nanP* and *plo fimA fimC fimE nanH* showed high frequencies, and the frequencies were all 3 (17.64%) isolates (Table 5).

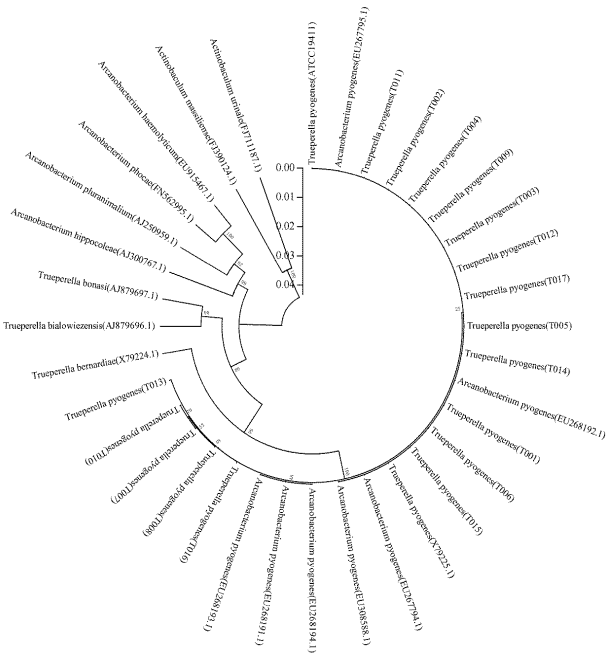

Fig. 1: Dendrogram analysis based on *16S rRNA* gene sequences among 17 *T. pyogenes* isolates and other related species achieved from the genetic database (NCBI GenBank).

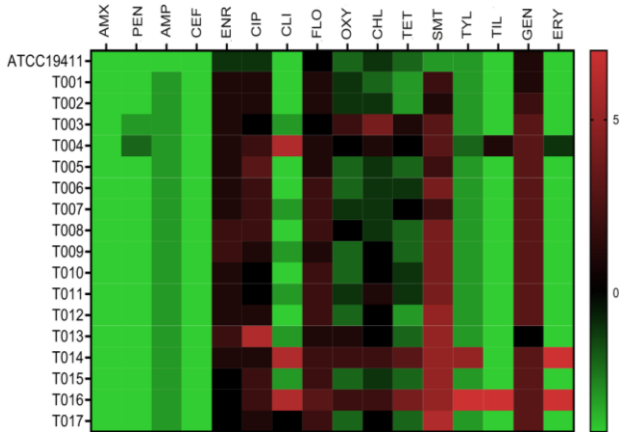

Fig. 2: MICs of all *T. pyogenes* isolates from dairy cows with mastitis. The colors present the value of log_2MIC of corresponding antimicrobial agents.

Table 3: MICs (μg/mL) of *T. pyogenes* from dairy cows with mastitis (17 isolates)

Antimicrobial agents	MIC (μg/mL)		Break point (μg/mL)	Resistant rate (%)
	MIC50	MIC90		
Amoxicillin	0.0625	0.0625	≥2	0(0/17)
Ampicillin	0.125	0.125	≥0.5	0(0/17)
Chlortetracycline	1	4	≥4	17.65%(3/17)
Cefotaxime	0.125	0.125	≥16	0(0/17)
Cefquinome	0.5	0.5	-	-
Ceftiofur	0.125	0.25	≥8	0(0/17)
Ciprofloxacin	2	4	≥4	47.06%(8/17)
Clindamycin	0.125	64	≥4	23.53%(4/17)
Enrofloxacin	1	4	≥2	17.65%(3/17)
Erythromycin	0.0625	0.5	≥1	11.76%(2/17)
Florfenicol	4	4	≥8	5.88%(1/17)
Gentamicin	8	8	≥16	0(0/17)
Oxytetracycline	0.5	16	≥8	23.53%(4/17)
Penicillin	0.03	0.03	≥0.06	0(0/17)
Sulfamethoxazole/ Trimethoprim	16	32	0.125	100%(17/17)
Tetracycline	0.5	2	≥8	5.88%(1/17)
Tylosin	0.125	16	-	-
Tilmicosin	0.0625	0.0625	≥0.5	11.76%(2/17)

Table 4: Resistance phenotype of 17 *T. pyogenes* isolates and its correlation with the occurrence of the integrons

Isolates	Resistance phenotype [a]	Detection of class I integrons[b]	Gene cassettes
T001	SMT	I	-[c]
T002	SMT	I	-
T003	CHL/OXY / SMT	I	-
T004	TIL/ CLI /CIP/ SMT	I	*aadA9*
T005	CIP/ SMT	I	*aadA9*
T006	CIP/ SMT	I	-
T007	CIP/ SMT	I	-
T008	CIP/ENR/SMT	I	-
T009	ENR/ SMT	I	-
T010	SMT	I	-
T011	SMT	I	-
T012	SMT	I	-
T013	OXY/ CIP/ENR / SMT	I	-
T014	CHL/OXY /ERY/ CLI /SMT	I	*aadA5-dfrA17*
T015	CIP/SMT	I	*aadA5-dfrA17*
T016	CHL/OXY /TET /TYL/ERY /TIL /CLI /FLO/CIP/SMT	I	*aadA5-dfrA17*
T017	CLI/ SMT	I	-

[a]Abbreviations for antimicrobial agents: CHL=chlortetracycline, OXY= oxytetracycline, TET=tetracycline, TYL=tylosin, ERY=erythromycin, TIL=tilmicosin, CLI=clindamycin, FLO=florfenicol, CIP=ciprofloxacin, ENR=enrofloxacin, SMT= Sulfadimethoxine/trimethoprim. b Only class I integrons were detected in *T. pyogenes* isolates, but no class II integrons appeared. [c] A dash indicates not detected.

Table 5: Genotypes of *T. pyogenes* isolates in this study (17 isolates)

Genotype	Isolates	
	Number	%
plo nanH nanP cbpA fimA fimC fimE	3	17.64
plo nanH nanP cbpA fimA fimC	3	17.64
plo nanH nanP fimA fimC fimE	1	5.88
plo nanH nanP cbpA fimA fimE	1	5.88
plo nanH nanP fimA fimC fimE	4	23.52
plo nanH cbpA fimA fimC fimE	1	5.88
plo nanH fimA fimC fimE	3	17.64
plo nanH fimA fimE	1	5.88

DISCUSSION

It is of importance for prudent use of antimicrobials both in humans and food-producing animals, which is essentially required to combat the increasing resistance. The MIC is normally determined using the Broth Microdilution Method according to CLSI (2017) and is helpful to adjust the medicine regimen to reach therapeutic concentrations. The β-lactams, such as penicillin and ampicillin, are usually used in veterinary medicine, all *T. pyogenes* isolates from dairy cow with mastitis are susceptible to these antimicrobial agents in our study; similarly, 97.8% of *T. pyogenes* isolates from pigs are susceptible to penicillin (Galán-Relaño *et al*., 2020b). However, our previous results indicated that *T. pyogenes* isolates from dairy cow with endometritis showed non-susceptible to these antimicrobial agents (Liu *et al*., 2009), and isolates from bovine mastitis and metritis showed nonsusceptible to peniclillin (100%) and amoxillin (87.5%) in Iran (Rezanejad *et al*., 2019). We believe that the preference of antimicrobial agents contributes to this, thus *T. pyogenes* isolates from dairy cows with mastitis showed susceptible to β-lactams including ampicillin and amoxicillin (de Boer *et al*., 2015; Pohl *et al*., 2018). But it is crucial to combine the resistance phenotype and the epidemiological or clinical interpretive criteria to explain these results.

The tetracyclines and tylosin were widely used as feed additives and growth promotion in food-producing animals, and this led to the selected pressure to resistance in pathogens, including *T. pyogenes* (Jost *et al*., 2004; Billington and Jost, 2006). Most isolates were susceptible to tetracyclines and tylosin in this study, but the frequency of this resistance was lower when compared with those in previous studies (Santos *et al*., 2010; Zastempowska and Lassa, 2012). The resistance of fluoroquinolone in *T. pyogenes* is rarely described by other researchers. However, we observed a high frequency in floroquinolone including ciprofloxacin and enrofloxacin, and similar results were detected in *T. pyogenes* isolates from European bison and forest musk deer (Zhao *et al*., 2011; Rzewuska *et al*., 2012). Further study should be carried out to investigate the factors contributing to fluoroquinolone resistance in *T. pyogenes*.

In this study, the results showed that aminoglycoside-resistance determinants (*aadA5* and *aadA9*) and trimethoprim-resistance determinants (*dfrA17*) were normally carried by class I integrons in *T. pyogenes* isolates. Interestingly, similar result was achieved from isolates from dairy cows in the Jilin Province of China (Dong *et al*., 2019), this phenomenon may attribute to the trade of animal husbandry between these two areas. At the same time, studies for other clinical infections indicated that aminoglycoside-resistance determinants (*aadA1*, *aadA2* and *aacC*), trimethoprim-resistance determinant (*dfr2a*) and β-lactam-resistance determinant (*blaP1*) were positive in *T. pyogenes* isolates (Zhao *et al*., 2011; Zastempowska and Lassa, 2012), which means *T. pyogenes* isolates can harbor a variety of antimicrobial resistance determinants.

All isolates were positive for *plo*, *nanH* and *fimA* genes, moreover, *fimC*, *fimE*, and *nanP* were harbored by most of *T. pyogenes* isolates in our study. Thus, we speculate that *plo*, *nanH*, and *fimA* play important roles in its pathogenesis when causing infection in udders of dairy cows. Similarly, reports from other researchers indicated that *plo* and *nanH* were detected among all *T. pyogenes* isolates from dairy cows with mastitis (Tamai *et al*., 2018; Rezanejad *et al*., 2019). The *cbpA* gene, which is associated with encoding collagen-binding protein, was habored by 47.06% isolates from dairy cows with clinical mastitis, however, comparable lower rates were reported by Zastempowska and Lassa (2012) and Rezanejad *et al*. (2019). *T. pyogenes* isolates from uterine infection generally harbored a high rate of *cbpA* with 48.9% (Santo *et al*. 2010).

From 185 milk samples, several pathogens are identified, such as *Staphylococcus aureus* (n=56), *Escherichia coli* (n=40), coagulase-negative staphylococci (n=32) and *Streptococcus uberis* (n=14). The antimicrobial resistance of these pathogens needs to be investigated in the future research, and this will give guidelines when choosing antimicrobial agents in treating clinical mastitis of dairy cows in Liaoning of China.

Conclusions: In conclusion, β-lactam antibiotics including amoxicillin, ampicillin, penicillin cefotaxime, cefquinome, and ceftiofur can be used in treating infections caused by *T. pyogenes*. In addition, *plo*, *nanH* and *fimA* gene were the genes positive among all the isolates, but other virulence genes were positive with various frequencies. Finally, measures should be taken to prevent class I integrons transmission between different bacteria. Our results will be helpful in preventing and treating clinical mastitis caused by *T. pyogenes* in dairy cows, and ultimately for reducing economic losses in dairy herds.

Authors contribution: YR Guo, P Qiu, MC Liu and DX Zhang designed and carried out experiments, and HY Su, Y Li, Y Guo, YC Liu collected samples and analyzed the data. DX Zhang and MC Liu wrote the manuscript, and all authors discussed the study. All authors approved the final version of this manuscript.

Acknowledgements: This research was supported by National Natural Science Foundation of China (No. 31772795 and 31972736).

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