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PROPERTIES AND ANTIMICROBIAL SUSCEPTIBILITY OF *TRUEPERELLA PYOGENES* ISOLATED FROM BOVINE MASTITIS IN CHINA

Rashad ALKASIR[1,†], Jianfang WANG[2,†], Jian GAO[1], Tariq ALI[1], Limei ZHANG[1], Ottó SZENCI[3,4], Árpád Csaba BAJCSY[3,4] and Bo HAN[1*]

[1]College of Veterinary Medicine, China Agricultural University, No. 2 Yuan Ming Yuan West Road, Haidian District, 100193 Beijing, PR China; [2]Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, Beijing University of Agriculture, Beijing, PR China; [3]Department and Clinic for Production Animals, Faculty of Veterinary Sciences, Szent István University, Üllő, Dóra major, Hungary; [4]MTA-SZIE Large Animal Clinical Research Group, Üllő, Dóra major, Hungary

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Trueperella (T.) pyogenes is an opportunistic pathogen that causes suppurative diseases in domestic animals. In this work, the properties, pathogenesis and phenotypic diversity of *T. pyogenes* isolates from bovine mastitis were studied. Both pyolysin *(plo)* and collagen-binding protein *(cbp)* virulence factor genes were detected by PCR in all *T. pyogenes* isolates (n = 50). Using the tissue culture plate method, 90% of *T. pyogenes* isolates were able to form biofilms. The minimum inhibitory concentrations (MICs) of 13 antimicrobials against *T. pyogenes* isolates were determined. High susceptibility was observed to rifampin (96%), ampicillin (94%), ciprofloxacin (94%), and penicillin (92%), while low susceptibility was found to trimethoprim–sulphamethoxazole (10%) and bacitracin (2%). The intracellular assay revealed that *T. pyogenes* isolates had different cytopathogenic effects on cells. The high percentage (28.6%) of *T. pyogenes* isolates suggests that this bacterium is an important contributor to mastitis. Moreover, the high occurrence of multidrug resistance, biofilm production, intracellular survival, and the temporal dynamics of *T. pyogenes* interactions are key factors for a better understanding of how immunity acts on infections with these bacteria and how they evade immune surveillance, thus highlighting the need for the prudent use of antimicrobial agents in veterinary medicine.

Key words: *Trueperella pyogenes*, bovine mastitis, biofilm, virulence factor, antimicrobial susceptibility

Mastitis is an inflammation of the mammary gland caused by several varieties of bacteria such as *Staphylococcus (S.) aureus*, *S. epidermidis,* and strep-

[†]Equal contributors
[*]Corresponding author; E-mail: hanbo@cau.edu.cn; Phone: 0086 (10) 6273-3801; Fax: 0086 (10) 6273-7865

tococci, as well as *Trueperella (T.) pyogenes*, formerly classified as *Arcanobacterium pyogenes* (Ramos et al., 1997; Yassin et al., 2011). *Trueperella pyogenes* is an opportunistic pathogen that causes suppurative diseases including mastitis, abscesses, pneumonia, and lymphadenitis in economically important food animals such as cows and sheep (Ribeiro et al., 2015). The presence of known potential virulence factors of *T. pyogenes,* such as the haemolytic exotoxin pyolysin *(plo)*, collagen-binding protein *(cbp),* and factors stimulating adhesion to host cells including fimbriae *(fimA, fimC, fimE, fimG)* and neuraminidases *(nanH, nanP),* has been studied in *T. pyogenes* strains (Silva and Lobato, 1999; Jost et al., 2002; Hijazin et al., 2011; Zhao et al., 2013). Bacterial biofilms, embedded in a self-produced polymer matrix consisting of polysaccharide, protein, and DNA, show an increased tolerance to antibiotics and disinfectants as well as resistance to phagocytosis and other components of the immune system; therefore, they result in chronic infections (Høiby et al., 2010). Previous studies showed that the intramammary administration of antibiotics against *T. pyogenes* infections was ineffective (Jousimies-Somer et al., 1996), whereas prolonged and intensive systemic treatment of early cases with beta-lactams and rifampin was occasionally effective (Hirvonen et al., 1994; Andrews et al., 2004; Zhang et al., 2014).

Nevertheless, little is known about bacterial and host factors that may contribute to the establishment and persistence of intramammary infection by *T. pyogenes* (Jost and Billington, 2005). In addition, most aspects of the pathogenesis of infection caused by *T. pyogenes* originating exclusively from bovine mastitis have been poorly characterised to date (Zastempowska and Lassa, 2012). The aim of this study was to explore the pathogenesis and antibiotic resistance of *T. pyogenes* strains isolated from bovine mastitis cases in China.

Materials and methods

Isolation and identification of T. pyogenes *from milk*

Milk samples (n = 175) from Chinese Holstein-Friesian dairy cows with clinical and subclinical mastitis were collected from four commercial dairy farms in Northern China (Beijing: n = 105; Hebei province: n = 35; Tianjin: n = 35) between June 2012 and July 2013. All samples were collected as described previously (Liu et al., 2014) with the consent of dairy herd owners.

The samples were cultured aerobically on Trypticase Soy Agar (TSA) (Difco™, Beijing, China) supplemented with 5% defibrinated sheep blood and on Mueller-Hinton Agar (MHA) plates (Difco™, Beijing, China) for 48 h at 37 °C. Colonies showing typical growth features for *T. pyogenes* (beta-haemolytic, Gram-positive and small, curved rod-shaped bacteria by microscopic examination) were selected for further investigations. All bacteriological examinations were performed under conventional aerobic conditions (Whitman et al., 2009). Syner-

gistic haemolytic activity of the isolates was determined in the Christie-Atkins-Munch-Petersen (CAMP) test, performed with the beta-haemolysin producing *S. aureus* strain ATCC 29213 on TSA with 5% sheep blood. Confirmation of suspected isolates was performed by amplification of the 16S rRNA gene followed by single-strand sequencing. The sequence data were compared with those of the GenBank database using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast/cgi), and homology levels ≥ 98% were considered adequate for species identification (Alexeeva et al., 2006). Subsequently, all *T. pyogenes* isolates were stored at −80 °C in the cryoprotective medium of Trypticase Soy Broth (TSB) (Difco™, Beijing, China) supplemented with a 25% volume of glycerol. One reference strain (*T. pyogenes* ATCC 19411) was included in the study.

Detection of potential virulence factor genes

PCR was used to detect genes encoding seven known and putative potential virulence factors of *T. pyogenes*. The primers and reaction conditions were previously described (Silva and Lobato, 1999), and used in this study with minor modifications. For each amplified gene, the thermal cycling conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 0.5 min, annealing for 1 min at a temperature specified by Silva and Lobato (1999), extension at 72 °C for 45 sec and, after the last cycle, a final extension at 72 °C for 7 min. Products of amplification were separated by electrophoresis through 1.5% agarose gel and stained with ethidium bromide.

Biofilm production

The capacity of *T. pyogenes* to form biofilms was assayed by the tissue culture plate (TCP) method (Hassan et al., 2011) with a slight modification. The experiment was performed in triplicate. Biofilm production was analysed according to the standards of a previous study (Stepanovic et al., 2007).

Antibiotic susceptibility

Antimicrobial susceptibility of the *T. pyogenes* strains (n = 50) to 13 different antibiotics (Oxoid™, Beijing, China) was determined using the minimum inhibitory concentration (MIC) techniques (Clinical and Laboratory Standards Institute, 2008). *Staphylococcus aureus* ATCC 29213 was used as a quality control organism. The results were read and summarised after 24-h incubation. Because there were no interpretive criteria specific for *T. pyogenes* in the CLSI document, the interpretive criteria of *S. aureus* were used instead.

Intracellular assessment of T. pyogenes

Two isolates of *T. pyogenes* showing the same biochemical activities and different biofilm production were selected, and the destiny of 25 and 100 bacteria/cell was adjusted to be used for the assay. The invasion assay was performed as described previously (Alkasir et al., 2012).

Statistical analysis

The statistical significance of the results was established using Fisher's exact test and the level of significance was set at $P < 0.05$. All experiments were performed in triplicate.

Results

Isolation and identification of T. pyogenes *from milk*

A total of fifty *T. pyogenes* isolates were recovered from 175 milk samples collected from four commercial dairy farms. This bacterium was the primary identified bacterial pathogen in 28 (56%) of the samples positive for *T. pyogenes* (Table 1). The isolates showed a colony morphology typical of *T. pyogenes*. All isolates formed small, circular, convex, opalescent and glistening smooth colonies (0.1–0.5 and 0.2–1.2 mm in diameter after 24 and 48 h, respectively), which were white with a narrow zone of beta haemolysis. In addition, the isolates failed to grow on MHA plates. The isolates were Gram positive, small, curved and rod shaped when viewed under the microscope, and differed in their haemolytic activity. Moreover, during the primary isolation and identification, few isolates of *T. pyogenes* showed differences in colony size after 48 h of incubation. However, the colony size became more uniform after subculture. In the CAMP test, a weak enhancement of staphylococcal haemolysis was observed for 10 strains (20%). The remaining isolates exhibited a strong synergistic effect in this test.

Genes encoding potential virulence factors

Data regarding potential virulence factor genes are presented in Table 2. All *T. pyogenes* isolates carried the *plo* and *cbpA* genes, whereas other potential virulence factor genes were found with different frequencies.

Biofilm production

Ninety percent (45 out of 50) of the examined *T. pyogenes* isolates were able to form biofilms, and the results of the OD_{570} presented three different groups. Eighteen isolates were classified as highly biofilm positive ($OD_{570} \geq 1$) (Group A). In addition, 27 isolates showed low-grade biofilm formation ($0.1 \geq OD_{570} < 1$) (Group B), and 5 isolates were biofilm negative ($OD_{570} < 0.1$) (Group C).

Table 1

Isolation of *Trueperella pyogenes* strains (n = 50) and other pathogens from mastitis cases in Chinese dairy cows

Dairy farm location	Mastitis cases (n)	*T. pyogenes* isolates (n)	*T. pyogenes* isolates individually recovered from mastitis cases [n (%)]	Other major pathogens found in the same samples
Beijing farm 1	60	20	11 (55%)	*Staphylococcus aureus,* Streptococcus group B, *Escherichia coli, Aerococcus viridans*
Beijing farm 2	45	10	5 (50%)	*S. aureus,* Streptococcus group B, *Nocardia asteroides, Prototheca zopfi*
Tianjin	35	9	5 (55.5%)	*S. aureus, E. coli, A. viridans*
Hebei	35	11	7 (63.6%)	*S. aureus,* Streptococcus group B, *Enterobacter aerogenes*

Table 2

Prevalence of virulence factor genes carried by *Trueperella pyogenes* isolates (n = 50) in various Chinese dairy farms

Farm location (n)	Virulence factor						
	plo	*cbpA*	*nanH*	*nanP*	*fimA*	*fimC*	*fimG*
Beijing farm 1 (20)	20	20	6	7	19	17	6
Beijing farm 2 (10)	10	10	4	3	10	8	3
Hebei (11)	11	11	6	5	10	10	5
Tianjin (9)	9	9	4	4	7	6	5
Total (50)	50	50	20	19	46	41	19

plo: pyolysin, *cbpA:* collagen-binding protein, *nan*: neuraminidases, *fim:* fimbriae

Antibiotic susceptibility

The sensitivity of *T. pyogenes* to 13 antibiotics is shown in Table 3. All *T. pyogenes* strains included in the current study were sensitive to rifampin (96%), ciprofloxacin (94%), and ampicillin (94%), whereas a low sensitivity was found to trimethoprim–sulphamethoxazole (10%) and bacitracin (2%).

Table 3

In vitro antimicrobial susceptibility of *Trueperella pyogenes* strains (n = 50) isolated from clinical mastitis cases*

Antimicrobial agent	Breakpoints (μg ml^{-1})			Susceptibility		MIC of ATCC 29213 (μg mL^{-1})	Acceptable QC ranges of MICs (μg mL^{-1})
	S	I	R	N	%		
Rifampin	≤ 1	2	≥ 4	48	96	0.004	0.004–0.015
Ampicillin	≤ 0.25	–	≥ 0.5	47	94	1	0.5–2
Ciprofloxacin	≤ 1	2	≥ 4	47	94	0.125	0.12–0.25
Penicillin	≤ 0.12	–	≥ 0.25	46	92	0.5	0.25–2
Azithromycin	≤ 2	4	≥ 8	40	80	1	0.5–2
Enrofloxacin	≤ 0.0625	–	≥ 1	40	80	0.125	0.12–0.25
Cefaclor	≤ 8	16	≥ 32	39	78	1	1–4
Erythromycin	≤ 0.5	1–4	≥ 8	28	56	0.25	0.25–1
Gentamicin	≤ 4	8	≥ 16	25	50	0.25	0.12–1
Clindamycin	≤ 0.5	1–2	≥ 4	25	50	0.0625	0.06–0.25
Tetracycline	≤ 4	8	≥ 16	15	30	0.125	0.12–1
Trimethoprim–sulphamethoxazole	≤ 2/38	–	≥ 4/76	5	10	0.25/4.75	≤ 0.5/9.5
Bacitracin	≤ 0.025	–	–	1	2	0.025	–

**Staphylococcus aureus* ATCC 29213 was used as quality control, according to the Clinical and Laboratory Standards Institute (2008). S: sensitive isolates; I: intermediate isolates; R: resistant isolates; QC: quality control; MIC: minimum inhibitory concentration; ATCC: American Type Culture Collection

Intracellular assessment of T. pyogenes

Following a 3-h invasion incubation period, BMEC cells that harboured two *T. pyogenes* isolates were incubated for 48 h in the presence of antibiotics. The number of intracellular CFU per millilitre recovered at 3 h was compared with the number recovered at 6 h, 12 h, 24 h, and 48 h for each bacterial density evaluated. Almost similar numbers of intracellular CFUs per millilitre were obtained at 3 h, 6 h and 12 h, while a decline towards the x-axis was observed at time points 24 h and 48 h, respectively. These results indicate that *T. pyogenes* remained viable within the BMEC cells but probably did not replicate. In addition, the *T. pyogenes* isolate which produce a high amount of biofilm showed longer intracellular survival than the isolate with lower biofilm production (Fig. 1).

Discussion

In this study, *T. pyogenes* strains isolated from bovine mastitis in dairy herds from different parts of China showed substantial phenotypic diversity. The most important morphological and physiological characteristics were the same as those described previously for *T. pyogenes* (Markey et al., 2013). The majority (80%) of the isolates displayed a strong positive effect in the synergistic CAMP test performed with *S. aureus* producing beta-haemolysin. However, variable results (36–100%) have been reported for this test previously (Ülbegi-Mohyla et al., 2009; Hijazin et al., 2011; Rzewuska et al., 2012).

Trueperella pyogenes can express a number of various pathogenicity-determining factors. Pyolysin *(plo)* is a major virulence factor, which is responsible for the lysis of host cells (Jost and Billington, 2005). The presence of the *plo* gene was demonstrated in all *T. pyogenes* strains of various origin (Silva and Lobato, 1999; Santos et al., 2010; Hijazin et al., 2011; Zhao et al., 2011). The *plo* gene was also detected in all of our *T. pyogenes* strains isolated from bovine mastitis cases. Collagen-binding protein (*Cbp*A) is a virulence factor necessary for adhesion to host cells (Esmay et al., 2003; Pietrocola et al., 2007). In the present study, the *cbpA* gene encoding this protein was detected in all isolates (100%), while it was reported to have been recovered from 21% of bovine mastitis isolates (Zastempowska and Lassa, 2012). In addition, two neuraminidases, H (*nanH*: 40%) and P (*nanP*: 38%) were found in this study. Previous studies (Jost et al., 2001) showed that all investigated *T. pyogenes* isolates recovered from different types of infection were positive for *nanH* activity, and 64.2% of these isolates harboured the *nanP* gene. Both the *nanH* and *nanP* genes also occurred in all isolates recovered from the uterus of dairy cows (Silva and Lobato, 1999). In contrast, other studies reported that the *nanH* gene was present only in 20.2% (Zastempowska and Lassa, 2012) and 87% (Hijazin et al., 2011) of isolates of bovine mastitis samples. Furthermore, 46 *T. pyogenes* isolates (92%) carried the

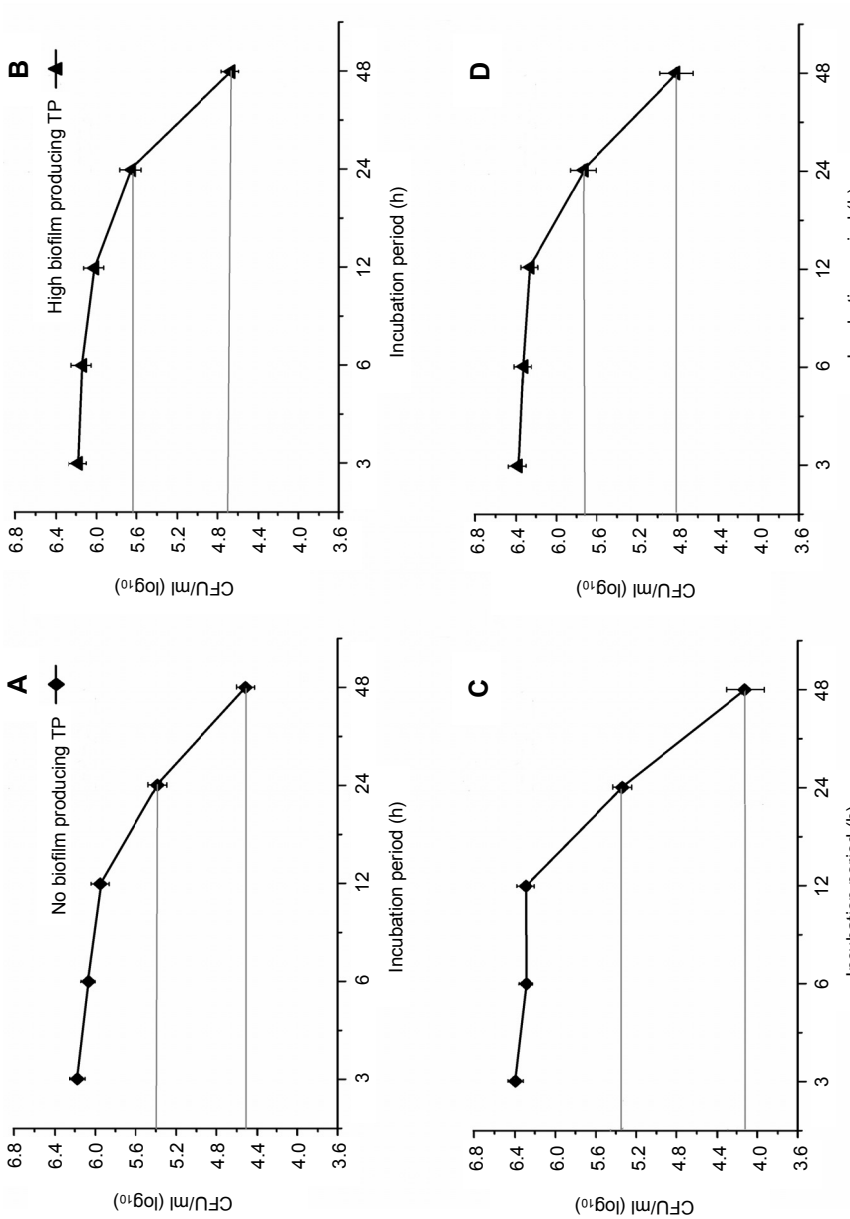

Fig. 1. Trueperella pyogenes (TP) [colony-forming units (CFU) ml^{-1}] recovered from BMEC cell line at several time points, with two different concentrations of bacteria/cell (A and B: 25/1, C and D: 100/1) of two different isolates [no biofilm producing TP (Group C): A and C, and high biofilm producing TP (Group A): B and D]. The number of intracellular CFU at each time point was determined in triplicate

fimA gene and most of them (82%) simultaneously harboured the *fimC* gene, while *fimG* was found in 38% of the isolates. These results are similar to the findings of a previous study (Silva and Lobato, 1999). The *fimC* gene seemed to occur more frequently in isolates from mastitis (88%) (Zastempowska and Lassa, 2012) than in those from metritis (67%) (Silva and Lobato, 1999). The *fimG* gene, in turn, was more prevalent among isolates from bovine metritis (50% or 67%) (Silva and Lobato, 1999; Santos et al., 2010) compared to those from bovine mastitis (18%) (Zastempowska and Lassa, 2012). Previous research has found that the expression of fimbriae plays an important role in the pathogenicity and biofilm formation of *Salmonella* Enteritidis and *E. coli* (Pruss et al., 2006; Soto et al., 2007; Lasaro et al., 2009; Zhu et al., 2013).

Trueperella pyogenes also produces protease and can form biofilms (Markey et al., 2013), which play an increasing role within the veterinary and clinical communities and are normally associated with the chronic nature of subsequent infections (Carvalhais et al., 2015). Jost and Billington (2005) showed that both haemolysin expression and biofilm production were upregulated by a common regulator named ploR, which may be a global regulator of *T. pyogenes* virulence. Our isolates were mostly susceptible to ciprofloxacin, rifampin, ampicillin, and penicillin, and were resistant or slightly susceptible to gentamicin, tetracycline, trimethoprim–sulphamethoxazole, and bacitracin. These results are in accordance with those of a previous study (Silva and Lobato, 1998), in which *T. pyogenes* was most sensitive to ampicillin, enrofloxacin, penicillin, gentamicin, and tetracycline. The high susceptibility of *T. pyogenes* to ampicillin and penicillin in this study is in contrast with the results of other authors (Malinowski et al., 2011).

Invasion of epithelial cells of the mammary gland can promote bacterial persistence by affording protection from host defence factors as well as antibiotics, and can provide a route to subepithelial tissues. Results from this study and from previous research (Jost and Billington, 2005) indicated that *T. pyogenes* invaded epithelial cells from the bovine mammary gland *in vitro*, which may be a common feature among pathogens associated with bovine mastitis.

The isolated *T. pyogenes* strains showed a high occurrence of multidrug resistance and biofilm production, which indicates that the resistance patterns of *T. pyogenes* have to be taken into consideration to enable a successful antibiotic treatment of clinical mastitis cases. However, little is known about the pathophysiology of *T. pyogenes* infection and its clinical importance in bovine mastitis. Therefore, further studies are needed in order to better understand the ability of this bacterium to invade and survive within epithelial cells and phagocytes, other putative potential virulence factors, and the role of *T. pyogenes* in the pathogenesis of mastitis.

In conclusion, the high percentage (28.6%) of *T. pyogenes* isolates suggests that *T. pyogenes* strains are important contributors to clinical mastitis.

Moreover, *T. pyogenes* showed high multidrug resistance, phenotypic diversity, biofilm formation, and intracellular survival, highlighting the need for the prudent use of antimicrobial agents in mastitis associated with *T. pyogenes*.

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