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ORIGINAL RESEARCH

Molecular Characterization of Resistance and Virulence Factors of *Trueperella pyogenes* Isolated from Clinical Bovine Mastitis Cases in China

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Purpose: The present study was designed to investigate the resistance determinants and virulence factors of 45 *Trueperella pyogenes* isolates from clinical bovine mastitis in Hexi Corridor of Gansu, China.

Methods: Minimum inhibitory concentrations (MICs) was tested by E-test method. Gene of antimicrobial resistance, virulence integrase and integron gene cassettes were determined by PCR and DNA sequencing.

Results: The T. pyogenes isolates exhibited high resistance to streptomycin (88.9%) and tetracycline (64.4%), followed by erythromycin (15.6%) and gentamicin (13.3%). Resistance to streptomycin was most commonly encoded by *aadA9* (88.9%); and to tetracycline, by *tetW* (64.4%). Importantly, all streptomycin-resistant isolates carried *aadA9* alone or in combination with *aadA1, aadA11* and *strA-strB*. Similarly, all tetracycline-resistant isolates harbored *tetW* alone or in combination with *tetA33*. Meanwhile, *ermX* was detected in 13.3% isolates, only one erythromycin-resistant isolate was not identified for this gene. Moreover, all *T. pyogenes* isolates carried class 1 integrons, and 17.8% of them contained gene cassettes, including arrays *aadA1-aadB* (4.4%), aad *A24-dfrA1-ORF1* (2.2%) and *aadA1* (2.2%). Furthermore, all tested isolates harbored virulent genes *plo* and *fimA*, followed by *fimC* (88.9%), *fimE* (86.6%) *nanP* (75.6%), *nanH* (40.0%), *cbpA* (35.6%) and *fimG* (6.7%).

Conclusion: To our knowledge, this is the first report of integron gene cassettes of T. pyogenes isolates from bovine mastitis cases in China. These findings are useful for developing the prevention and the virulence factors of *T. pyogenes* could be promising candidates for vaccine antigens for bovine mastitis caused by *T. pyogenes* in China.

Keywords: *Trueperella pyogenes*, resistance, integrons, gene cassette, bovine mastitis

Introduction

Bovine mastitis, mainly caused by bacterial pathogens, is the most prevalent and costly diseases of dairy industry worldwide.[1] Current control and prevent programs based on post-milking teat disinfection, culling of chronically affected cows and antibiotic therapy have successfully reduced the incidence of contagious pathogens mastitis.[2] Antibiotic treatment may lead to an increase in resistance of some environmental pathogens.[3] *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) is an important environmental pathogen causing mastitis, which is often described as an opportunistic pathogen in domestic animals worldwide.[4–7] This bacterium commonly cause persistent infection and pyogenic lesions in mammary gland, thus it is more often linked to the mastitis with a high severity of symptoms.[8–10]

Antimicrobial therapy is the primary measure of treatment for *T. pyogenes* infections,[11] According to reports, plant secondary metabolites, antimicrobial peptides, and bacteriophages are currently widely studied alternatives to antibiotics and have made significant progress.[12–14] Unfortunately, these antibiotic substitutes still need time to follow suit in drug development. However, the therapeutic effectiveness has been attenuated by emerging resistant strains result of the widespread use of antimicrobials in food animals.[15] Antimicrobial resistance of *T. pyogenes* is mainly attributed to different resistant genes, such as *tetW* (tetracyclines resistance), *ermB* and *ermX* (macrolides resistance) and *aacC,*

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aadA1, aadA9 and *aadA11* (aminoglycosides resistance), which pose a potential threat to both animal and human medicine due to their dissemination through mobile genetic elements.[7,16] Integrons are the common bacterial genetic elements that can capture, rearrange, and express mobile gene cassettes.[17] *T. pyogenes* integrons, especially classes 1 integrons, play important roles in the horizontal transfer of resistance genes.[18] Moreover, several known and putative virulence factors that may contribute to the pathogenicity of *T. pyogenes*, including pyolysin (Plo) with cytolytic activity on immune cells and adhesion-related factors collagen-binding protein (CbpA), neuraminidases (NanH and NanP), and fimbriae (FimA, FimC, FimE and FimG).[19] The genotypic profiles of these virulence factors varied greatly among *T. pyogenes* isolates in different infection.[20]

Although investigations on *T. pyogenes* were reported worldwide, little is known about the isolates originated exclusively from bovine mastitis in China. The aim of the current study was to investigate the resistance determinants and virulence genes of *T. pyogenes* isolated from clinical bovine mastitis cases in Gansu, China. To the best of our knowledge, this is the first description of integron gene cassettes of *T. pyogenes* isolated from bovine mastitis in China.

Materials and Methods

Bacterial Isolates

The 45 *T. pyogenes* strains investigated in this study were isolated from clinical bovine mastitis cases in 16 commercial dairy herds located in Hexi Corridor of Gansu in China during July 2000 to Aug 2022 and preserved in our laboratory. Mastitis infection was confirmed by the California Mastitis Test. After transportation to the laboratory, mastitic milk samples were inoculated onto blood agar plates supplemented with 5% defibrinated sheep blood and incubated with 5% CO_2 at 37°C for 48 h. Smooth and glistening bacterial colonies surrounded by a conspicuous β-hemolytic zone were further identified by PCR and sequencing as described in our previous study[21,22] (Figure 1).

The 16S rRNA gene was amplified by the 16S rDNA Bacterial Identification PCR Kit (Takara, Shiga, Japan) in accordance with the manufacturer's recommendations (https://www.takarabiomed.com.cn/DownLoad/RR176.pdf). The

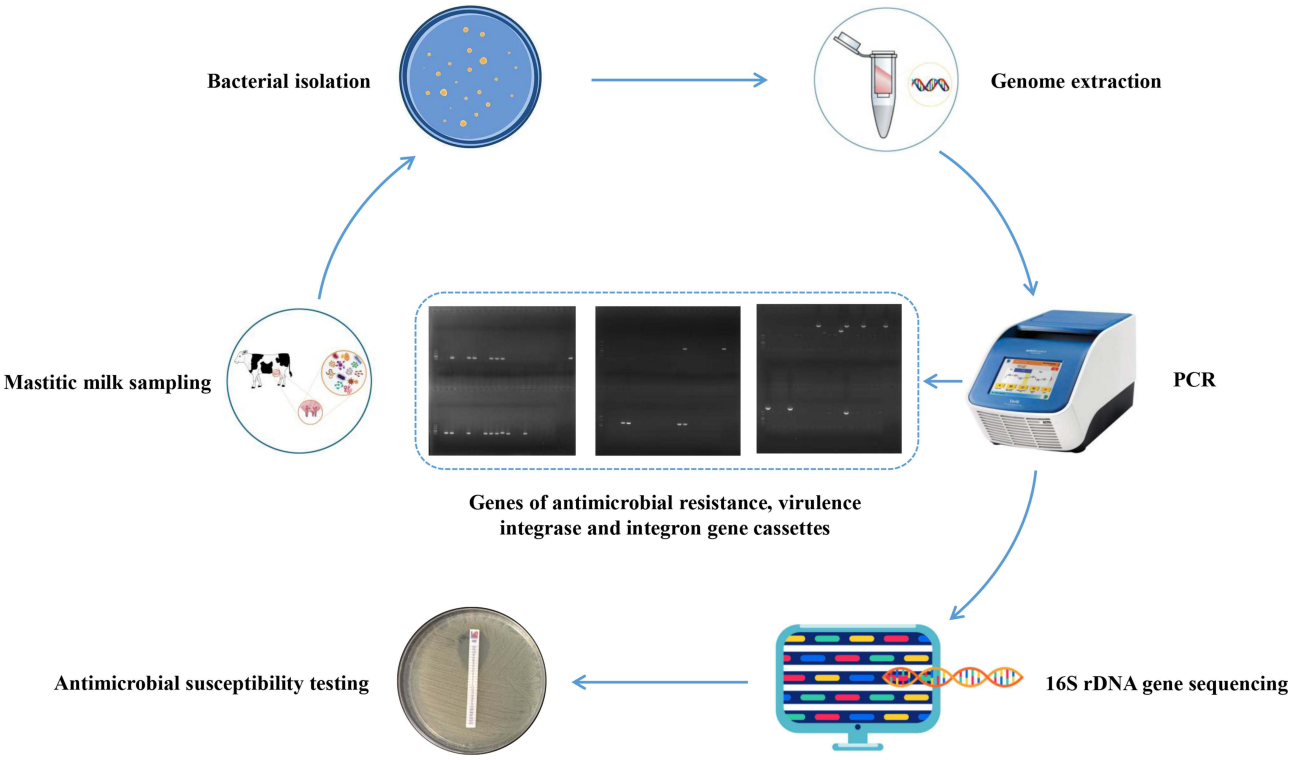

Figure 1 Experimental method flowchart.

PCR products were purified and sequenced by Sanger sequencing by Sangon Biotech (Shanghai) Co., Ltd. in China. Nucleotide sequences were analyzed with the program NCBI-BLAST (http://www.ncbi.nlm.nih.gov).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of penicillin, oxacillin, ceftiofur, erythromycin, tetracycline, streptomycin, gentamicin and ciprofloxacin against *T. pyogenes* were tested by E-test (Liofilchem, Roseto, Italy) method on Muller-Hinton agar supplemented with 5% defibrinated sheep blood. Antimicrobial agent concentrations ranged from 0.002 to 32 μg/mL for penicillin and ciprofloxacin, 0.016 to 256 μg/mL for oxacillin, ceftiofur, erythromycin, tetracycline and gentamicin, and 0.064 to 1024 μg/mL for streptomycin. *T. pyogenes* ATCC19411 was used as quality control strain. The experiments were carried out in triplicates biological replicate (three independent cultures). Currently, there are no *T. pyogenes*-specific breakpoints for antimicrobial susceptibility testing available in the Clinical and Laboratory Standards Institute guidelines. Thus, the susceptibility of the *T. pyogenes* was determined according to the breakpoints reported previously[23,24] (Figure 1).

Detection of Resistance Determinants and Virulence Genes

Single PCR was used to detect resistance genes of tetracyclines (*tet*W, *tet*A33, *tet*L, *tet*M, *tet*O, *tet*K and *tet*32), macrolides (*erm*X and *erm*B), and aminoglycosides (*aad*A1, *aad*A9, *aad*A11, *aac*C, *str*A-*str*B, *aph*(3')-IIIa and *aac*(6')-*aph*(2")), as well as integrase genes (*intI* I and *intI* II) and gene cassette region.[7,25–27] Briefly, the genomic DNA was extracted using the Bacterial DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The PCR products were analyzed using 1.0% agarose gel electrophoresis. Subsequently, DNA sequencing was carried out on gene cassette region. The data analysis of the gene cassettes was similar to that of the 16S rRNA gene. Similarly, genes encoding virulence factors pyolysin (*plo*), neuraminidases (*nanH* and *nanP*), collagen binding protein (*cbpA*) and fimbriae (*fimA, fimC, fimE* and *fimG*) were also determined by single PCR as previously described[7] (Figure 1).

Statistical Analysis

All drug resistance assays were carried out in triplicate. Data were classified using the Microsoft Office Excel software.

Results

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of the 45 *T. pyogenes* isolates against 8 antimicrobial agents were summarized in Table 1. The isolates showed high resistance to streptomycin (88.9%) and tetracycline (64.4%), followed by erythromycin (15.6%) and gentamicin (13.3%). Surprisingly, the MICs of erythromycin were greater than 256 μg/mL in 71.4% of the erythromycin-resistant isolates (data not shown). In addition, all tested isolates were susceptible to penicillin, oxacillin, ceftiofur and ciprofloxacin. It is worth noting that in this study, 20% of the isolates displayed multidrug resistance (MDR).

Genetic Determinants for Antimicrobial Resistance

In the present investigation the *T. pyogenes* isolates only showed resistance to tetracycline, erythromycin, gentamicin, and streptomycin. Hence, the corresponding resistant-genes of tetracyclines, macrolides and aminoglycosides as well as integrase genes and gene cassette region were tested and shown in Table 1. The *tet*W, *tet*A33, and *tet*K were found in 64.4%, 8.9% and 2.2% of the *T. pyogenes* isolates, respectively. All tetracycline-resistant isolates harbored *tet*W alone or in combination with *tet*A33. Besides, the erythromycin-resistant gene *erm*X was found in 13.3% of the isolates, one erythromycin-resistant isolate was negative for this gene. In addition, *aad*A1, *aad*A9, *aad*A11 and *str*A-*str*B were detected in 17.8%, 88.9%, 11.1%, and 11.1% of the tested isolates, respectively. Importantly, all streptomycin-resistant isolates were positive for *aad*A9 alone or in combination with *aad*A1, *aad*A11 or *str*A-*str*B. However, *erm*B, *tet*M, *tet*O, *tet*L, *tet*32, *aac*C, *aph*(3')-IIIa and *aac*(6')-*aph*(2") were not detected in any of the isolates. The results of integrase genes showed that all *T. pyogenes* isolates carried class 1 integron while no isolate harbored class 2. Furthermore, 17.8% of them were positive for gene cassettes, including arrays *aad*A1-*aad*B (8.9%), *aad*A2-*aad*B (4.4%), *aad*A24-*dfr*A1-ORF1 (2.2%) and *aad*A2 (2.2%). Notably, all gentamicin-resistant isolates contained gene cassette *aadB*.

Table 1 Distribution of Resistance Determinants and Virulence Genes in 45 *T. Pyogenes* Isolates from Clinical Bovine Mastitis

Resistance Pattern[a]	Resistance Genes	Inserted Gene Cassettes	Virulence Genes	No. of Isolates
TET, ERM, STR	*tet*W, *erm*X, *aad*A1, *aad*A9, *aad*A11, *str*A-*str*B	*aad*A2	*plo, nanH, nanP, fimA, fimG, fimE, fimC*	1
STR	*aad*A9		*plo, nanH, nanP, fimA, fimG, fimE, fimC*	1
TET, ERM, GEN, STR	*tet*W, *tet*A33, *erm*X, *aad*A1, *aad*A9, *aad*A11	*aad*A1-*aad*B	*plo, nanH, nanP, fimA, fimE, fimC*	1
STR	*aad*A9		*plo, nanH, nanP, fimA, fimE, fimC*	4
TET, GEN, STR	*tet*W, *aad*A1, *aad*A9	*aad*A1-*aad*B	*plo, nanP, cbpA, fimA, fimE, fimC*	1
TET, STR	*tet*W, *aad*A9, *str*A-*str*B		*plo, nanP, cbpA, fimA, fimE, fimC*	3
TET, STR	*tet*W, *aad*A9		*plo, nanP, cbpA, fimA, fimE, fimC*	8
TET	*tet*W		*plo, nanP, cbpA, fimA, fimE, fimC*	1
STR	*tet*A33, *aad*A9		*plo, nanP, cbpA, fimA, fimE, fimC*	1
	*tet*K		*plo, nanP, cbpA, fimA, fimE, fimC*	1
STR	*aad*A9		*plo, nanP, fimA, fimG, fimE, fimC*	1
STR	*aad*A9		*plo, nanH, nanP, cbpA, fimA*	1
TET	*tet*W		*plo, nanH, nanP, fimA, fimE*	1
TET, GEN, STR	*tet*W, *aad*A1, *aad*A9, *str*A-*str*B	*aad*A1-*aad*B	*plo, nanH, nanP, fimA, fimC*	1
TET, STR	*tet*W, *aad*A9		*plo, nanH, nanP, fimA, fimC*	1
STR	*aad*A9		*plo, nanH, fimA, fimE, fimC*	4
TET, STR	*tet*W, *aad*A9		*plo, nanP, fimA, fimE, fimC*	3
STR	*aad*A9		*plo, nanP, fimA, fimE, fimC*	1
TET	*tet*W		*plo, nanH, fimA, fimE*	1
STR	*aad*A9		*plo, nanH, fimA, fimE*	1
TET, ERM, GEN, STR	*tet*W, *erm*X, *aad*A1, *aad*A9	*aad*A1-*aad*B	*plo, nanH, fimA, fimC*	1
TET, ERM, STR	*tet*W, *aad*A1, *aad*A9, *aad*A11	*aad*A24-*dfr*A1-ORF1	*plo, nanP, fimA, fimE*	1
TET, STR	*tet*W, *aad*A9		*plo, nanP, fimA, fimC*	1
STR	*aad*A9		*plo, nanP, fimA, fimC*	1
TET, ERM, GEN, STR	*tet*W, *erm*X, *aad*A1, *aad*A9, *aad*A11	*aad*A2-*aad*B	*plo, fimA, fimE, fimC*	1
TET, GEN, STR	*tet*W, *aad*A1, *aad*A9, *aad*A11	*aad*A2-*aad*B	*plo, fimA, fimE, fimC*	1
TET, ERM, STR	*tet*W, *tet*A33, *erm*X, *aad*A9		*plo, fimA, fimE, fimC*	1
TET, ERM	*tet*W, *tet*A33, *erm*X		*plo, fimA, fimE, fimC*	1

Abbreviations: [a]TET, tetracycline; ERY, erythromycin; GEN, gentamicin; STR, streptomycin.

Detection of Virulence Genes

We also detected the virulence-encoding genes of the *T. pyogenes* isolates (Table 1). The results showed that all tested isolates carried *plo* and *fimA*. Genes *fimC, fimE, nanP, nanH* and *cbpA* were found in 88.9%, 86.6%, 75.6%, 40.0%, and 35.6% the isolates, respectively. While only 6.7% of the isolates contained *fimG*. In addition, a total of 14 different virulent genotypes were identified in the isolates. *Plo, nanP, cbpA, fimA, fimE* and *fimC* was the most dominant genotype and was detected in 33.3% of the isolates.

Discussion

T. pyogenes is an important opportunistic animal pathogen causing a wide variety of purulent infections and conveys significant economic losses to the animal husbandry industry. Beta-Lactams, tetracyclines and macrolides were often used to treat *T. pyogenes* infections, which accelerated the emergence of resistant strains.[28] In this study, most of the *T. pyogenes* isolates displayed high resistance against streptomycin and tetracycline and a few of them were resistant to erythromycin or gentamicin. Similar phenotypic resistance to these antimicrobial agents had been frequently observed in *T. pyogenes* isolates result from frequent use of these antimicrobials.[23,29–31] Bacterial tetracycline resistance is mainly conferred by *tet* genes of the ribosomal protection class, particularly the widespread determinant *tet*W.[32] Indeed, in the current study, all tetracycline-resistant isolates carried the *tet*W. Although few of the resistant isolates also contained tetracycline-specific efflux pump protein-encoding gene *tet*A33, the positive isolates are usually not considered resistant because of the low tetracycline MIC conferred by *tet*A33 alone in *T. pyogenes*.[25] These results

suggesting tetracycline-resistant mechanism was closely related to ribosomal protection proteins encoded by the *tet*W in *T. pyogenes* isolated from clinical bovine mastitis cases in Hexi Corridor of Gansu, China.

Although of the low erythromycin-resistant frequency (7/45) in the tested isolates, most of the *erm*X-containing isolates showed high erythromycin MICs (>256). Meanwhile, the MICs of erythromycin for the *erm*X-containing isolates varied considerably (data not shown). Moreover, one erythromycin-resistant isolate carried no expected resistance genes. This discrepancy could be attributed to the presence of other resistance mechanisms, such as additional *erm* genes, genes encoding efflux pumps, or ribosomal mutations.[33] In accordance with the previous report,[23] we found most of the *T. pyogenes* isolates were highly resistant to streptomycin but were susceptible to gentamicin. The *aad*A9 was the most prevalent aminoglycosides resistant-gene in the current study. This may not be surprising because *aad*A9 was shown to be significantly more prevalent in bovine isolates than other origin species.[26] Noteworthily, all streptomycin-resistant isolates carried *aad*A9 alone or in combination with *aad*A1, *aad*A11, or *str*A-*str*B, which mainly conferred to resistance against streptomycin.[26] Similarly, all gentamicin-resistant isolates were positive for class 1 integrons gene cassette *aad*B conferred to gentamicin resistance.[34] These results indicating that gene *erm*X and determinants *aad*A9 and gene cassette *aad*B may play an important role in erythromycin and aminoglycosides resistance in the tested isolates, respectively. Recently, more than 9 classes of integrons have been described, class 1 and 2 integrons are the most predominantly associated with antibiotic resistance in clinical isolates.[35,36] In current study, all *T. pyogenes* isolates carried class 1 integrons while no isolate harbored class 2. These data are in agreement with previous reports in China showed that class 1 was the most popular integrons in *T. pyogenes* isolates.[23,24] Additionally, 17.8% of the tested isolates were positive for 4 types of gene cassettes. All resistance gene cassettes were conferred resistance to aminoglycosides (*aad*A1-*aad*B, *aad*A2-*aad*B, *aad*A2 and *aad*A24) expect one trimethoprim-resistant gene cassette *dfr*A1, coinciding with previous study reported that these aminoglycosides resistance determinants were highly prevalent among pathogens from bovine mastitis in China.[37] It is worth noting that the high-level of aminoglycosides-resistant gene cassettes may facilitates horizontal transfer of these resistance genes among microorganisms,[35] which means that multidrug resistance can develop.[27]

T. pyogenes produces a number of extracellular or surface-exposed proteins involved in the infections caused by this bacterium.[38] Plo is a primary virulence factor with cytolytic activity related to transmembrane pore formation and considered as an important marker in the definitive diagnosis of *T. pyogenes*.[39,40] Similar to previous reports of bovine mastitis conducted in China and other countries,[6,7,11,29] *plo* was observed in all tested isolates, indicating it's a critical role in establishment of *T. pyogenes* infections. Other putative virulence factors in this study primarily contribute to the adhesion and colonization of the host tissues. Among them, cbpA is collagen-binding protein in *T. pyogenes* that mediates adhesion to epithelial and fibroblast cells.[41] Previous studies showed that the detection rate of *cpbA* ranged from 1.4% to 100.0% in *T. pyogenes* from bovine origins.[42–44] In this study, only 35.6% of the isolates harbored *cpbA*, which was much lower than other study in China reported that all tested isolates from bovine mastitis contained this gene.[11] Moreover, neuraminidases NanH and NanP were found to play an important role in the colonization of host tissue by cleaving the terminal sialic acid residues of host cell and reducing mucus viscosity of tissue.[45] In current study, the *nanH* and *nanP* genes were detected in 75.6% and 40.0% of the isolates, respectively. These findings are in accordance with the previous results of bovine *T. pyogenes* isolates.[39,46] The fimbriae were also involved in the cell adhesion and the colonization of host tissue. FimA is a dominating fimbria in *T. pyogenes*.[40] Indeed, all *T. pyogenes* isolates carried the *fimA* in this study, and most of them simultaneously harbored the *fimC* (88.9%) and *fimE* (86.6%). On the contrary, the *fimG* was only found in 6.7% of the isolates. These results were in accordance with the previous study that reported a high prevalence of *fimA* among *T. pyogenes* isolates, whereas other fimbriae-encoding genes were detected with different frequencies.[40] The differences of virulence factor genes detected in the current study could be explained by inherent variations between different isolates.[47]

Conclusions

The *T. pyogenes* isolates showed high frequencies of phenotypic and genotypic resistance to streptomycin and tetracycline, as well as high incidences of class 1 integrons and aminoglycosides-resistant gene cassette arrays among the cassettes, which remind the government to pay continuous attention to use antimicrobial agents in dairy industry. It is

worth noting that in this study, 20% of the isolates displayed multidrug resistance. Meanwhile, the potential threat of horizontal transmission of the resistance genes cannot be ignored. In addition, frequent occurrence of *plo, fimA, fimC, fimE* and *nanP* may indicate their pathogenic potential in bovine mastitis in China, although different infections caused by *T. pyogenes* may be equipped with variable virulence factors. Further investigations need to be performed to explore the diversity of virulence factors combination in *T. pyogenes* pathogenesis. In addition, in order to address the durability of antibiotics, we should further study the antibacterial mechanisms and effects of alternative to antibiotic.

Ethics Approval and Consent to Participate

Compliance with ethical standards: This study was approved by Ethics Committee of Gansu Agricultural University (No. GASU-Eth-AST-2023-008.) and was conducted in compliance with ethical, legal, and regulatory norms. The animal owners were informed about the purpose of the study, and consent of each animal owner was obtained before the physical examination of cows for clinical mastitis and the collection of milk samples.

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Disclosure

The authors report no conflicts of interest in this work.

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