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Prevalence assessment of *Staphylococcus aureus* and *Streptococcus agalactiae* by multiplex polymerase chain reaction (M-PCR) in bovine sub-clinical mastitis and their effect on somatic cell count (SCC) in Iranian dairy cows

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The contagious pathogens causing mastitis were evaluated by multiplex polymerase chain reaction (M-PCR) in 4 quarters of the first, third, and fifth parities in industrial, semi-industrial, and traditional dairy cattle farms in Isfahan province, Iran. Prevalence of sub-clinical mastitis caused by contagious pathogens in industrial, semi-industrial and traditional farms was 13.4, 38.2 and 44.2%, respectively, *Staphylococcus aureus* (*S. aureus*) was more prevalent than *Streptococcus agalactiae* (*S. agalactiae*) in all the dairy farms. However, prevalence of *S. agalactiae* was more in industrial farms as compared to traditional farms. The mean somatic cell count (SCC) did not differ significantly in different farm types. *S. aureus* was more prevalent than *S. agalactiae* in each parity number. Mean SCC between M-PCR⁻ and M-PCR⁺ status had no significant difference within the first parity. The mean SCC differed significantly with increasing parity number. In M-PCR⁻ status parity number had non-significant effect on mean SCC. Therefore, mean SCC was affected not only by pathogen type and diversity but also by parity number.

Key words: Multiplex polymerase chain reaction (M-PCR), somatic cell count, *Staphylococcus aureus*, *Streptococcus agalactiae*, contagious pathogen.

INTRODUCTION

Inflammation of the bovine udder is usually caused by bacterial infection, however it may be the result of sterile inflammation due to chemical, physical or mechanical trauma (Zadoks et al., 2002). The mastitis causing

pathogens are categorized as contagious and environmental pathogens. Among the contagious pathogens, the most common are *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*). They spread from infected to clean udder during the milking process through contaminated milker's hand, cloth towels and by flies (Allore, 1993). *S. aureus* is often cause by chronic mastitis and attributes to high somatic cell counts (SCCs) and damage to the secretory mammary epithelial cells. New infections are controlled by adopting measures like proper milking procedures, improved milking hygiene and housing management (Arnold, 2011). *S. agalactiae* continues to be a major

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Abbreviations: SCC, Somatic cell count; M-PCR, multiplex-polymerase chain reaction; *S. aureus*, *Staphylococcus aureus*; *S. agalactiae*, *Streptococcus agalactiae*.

cause of sub-clinical mastitis in dairy cattle, it is associated with elevated SCC and total bacteria count and a decrease in the quantity and quality of milk products produced (Keefe, 1997). Sub-clinical mastitis is important due to the fact that it is 15 to 40 times more prevalent than the clinical form, is of long duration, difficult to detect and adversely affects milk quality. Losses due to mastitis may even be higher in developing countries because standard mastitis control and prevention practices recommended by national mastitis council of USA are not being carried out promptly (Sharif and Muhammad, 2008). The SCC in bovine milk is an indicator of udder health and milk quality, commonly used to detect sub clinical mastitis. An increase in the SCC >200,000/ml suggests inflammation of the udder (Leitner et al., 2000; Kelly et al., 2000; Paape et al., 2003). Pathogens are generally isolated by conventional culture on selective media and further subjected to biochemical and serotyping tests. These procedures are cumbersome and time-consuming and often fail to detect bacteria in submitted samples from sub-clinically infected cows. In order to overcome the limitations of cultural methods, multiplex-polymerase chain reaction (M-PCR) has been developed to identify several various mastitis-causing pathogens simultaneously. Furthermore, the presence of pathogens can be demonstrated at an earlier stage of infection, in carrier animals and also negate false negative results caused by lack of bacterial growth (Ghorbanpoor et al., 2007; Aslam et al., 2003; Phuektes et al., 2003). Purpose of this study was to determine prevalence of contagious pathogens by M-PCR and the effect of pathogen type on mean SCC in dairy farms managed by various system of management in different parities from Isfahan province, Iran.

MATERIALS AND METHODS

Selection of cows

The study began by identifying the various farming system like, industrial and semi-industrial (medium) with >1000 and 21 to 100 cows farms cows, equipped with milking systems and hygienically maintained respectively, traditional (small) farms with ≤ 20 cows that were not equipped with milking systems. First, third, and fifth parity cows in each farm were identified. In industrial farms, cows were screened on the basis of CMT positive reactions, selection criteria being cow positive for sub clinical mastitis six months prior to the beginning of the present study. In semi-industrial and traditional farms final target cows were selected based on CMT positive reaction at the time of testing. A total of 288 samples ($3 \times 3 \times 4 \times 8 = 288$ according to farm type, parity, number of quarters, and number of replications, respectively) were obtained from 72 target Holstein cows with positive CMT results. Seventy-two Holstein cows were also selected from animals in the intermediate lactation period (75–100 days postpartum), and these cows were not treated with antibiotics 7 days prior to milk collection (Mallard et al., 1998; Gonzalo et al., 1996; Fuertes et al., 1998).

Collection of milk samples

Udders and teats were cleaned with warm water, left to dry, and

wiped with cotton buds soaked in 70% alcohol. Samples were aseptically collected in sterile tubes and transported to a veterinary and biotechnology research center and stored at 0 and -20°C for SCC and M-PCR, respectively (AOAC, 2002: 925.20) within 6 to 12 h of collection. Qualitative and quantitative analysis of SCCs were carried out (FDA, 2003; Wu, 2004), and M-PCR was performed (Henegariu et al., 1997).

Somatic cell counting

A total of 10 μL of homogenized sample was placed on a $1 \times 1 \text{ cm}^2$ surface of a microscopic slide and dispersed. The smear was air dried, kept in xylol for 2 to 5 min until the milk fat dissolved, air dried again, fixed with 95% ethanol for 2 to 5 min, and rinsed again. The sample was then immersed in 10% Giemsa solution (Lindmark-Mansson et al., 2006) for 3 to 5 min, washed in distilled water ($37-40^{\circ}\text{C}$), and immediately air dried. Cells with a nucleus were counted under a light microscope at $1000\times$ magnification according to the method described by Wu et al. (2004), FDA (2003), and IDF (1991).

DNA extraction

The genomic DNA of *S. aureus* and *S. agalactiae* was extracted from milk samples using DNPTM Kit (CinnaGen, Iran), according to the manufacturer's protocol. The isolated DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell (2001). The extracted DNA of each sample was kept frozen at -20°C until used (Berri et al., 2003).

M-PCR assay

In present study the oligonucleotide primers were designed using Primer3 software (version 1.1.0) for detection of *S. agalactiae* according to the published sequence (Accession no: FJ555494). The primers described by Phuektes et al. (2001) were used for detection of *S. aureus*. The M-PCR reaction was performed in a total volume of 25 μL in 0.5 ml tubes containing 1 μg of genomic DNA, 1 μM of each primer (primer details are presented in Table 1), 2 mM MgCl_2 , 200 μM dNTP, 2.5 μL of 10X PCR buffer and 1 unit of *Taq* DNA polymerase (Roche Applied Science, Germany). PCR amplification was carried out as follows: an initial denaturation at 95°C for 5 min; 32 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. PCR-amplified products were examined by agarose gel electrophoresis in a 2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide in TBE buffer. After electrophoresis, DNA was visualized by means of UV illumination (Henegariu et al., 1997). M-PCR was used to determine pathogen diversity, that is, M-PCR⁻ (absence of major pathogen), M-PCR⁺¹ (presence of one major pathogen) and M-PCR⁺² (presence of two major pathogens, simultaneously) in raw milk samples (Figure 1).

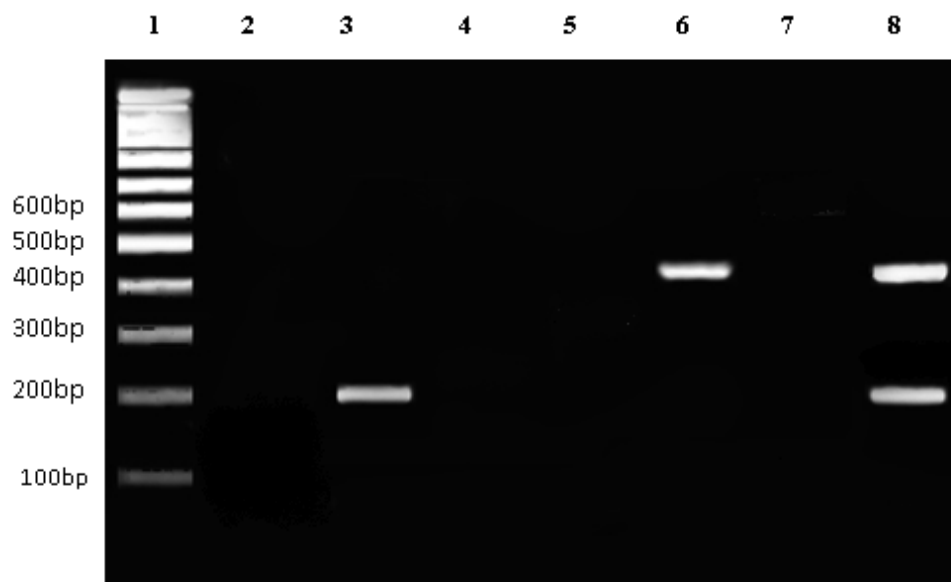
Statistical analysis

Statistical analysis was carried out to compare the prevalence udder infection, pathogen type, SCC, between the farming systems and parity numbers, using the SPSS (version 16) program. The total SCC data were analyzed by one-way analysis of variance (ANOVA), and group means were compared by Duncan's test. The effect of presence of one (M-PCR⁺¹) and two (M-PCR⁺²) contagious pathogens on mean SCC in raw milk samples was compared to M-PCR⁻ status (the absence of major pathogens in raw milk samples).

Table 1. Oligonucleotide primers for *S. aureus* and *S. agalactiae*.

Bacteria	Sequences of primers(5' → 3')	PCR product	Source
<i>S. aureus</i>	F: TCTTCAGAAGATGCGGAATA R: TAAGTCAAACGTTAACATACG	420 bp	Phuektes et al. (2001)
<i>S. agalactiae</i>	F: TAGTTTTGAGAGGTCTTGTGG R: ATATTCACAGCGTTTTTCG	206 bp	*Accession no: FJ555494

*Our design.

**Figure 1.** M-PCR products amplified from infected cows milk (Lanes 2-8). Lane 1: 100 bp molecular marker, Lane 2, 4, 5 and 7 (M-PCR⁻): negative *S. aureus* and *S. agalactiae*, Lane 3 (M-PCR⁺¹): positive *S. agalactiae* (206 bp); Lane 6 (M-PCR⁺¹): positive *S. aureus* (420 bp) Lane 8 (M-PCR⁺²): positive *S. aureus* (420 bp) and *S. agalactiae* (206 bp), simultaneously.

RESULTS

Prevalence of sub-clinical mastitis caused by contagious pathogens in industrial, semi-industrial and traditional farm was 13.4, 38.2 and 44.2%, respectively (Table 2). As in each farm *S. aureus* was more prevalent than *S. agalactiae*. Table 2 data revealed that when contagious and environmental pathogens were not detected by M-PCR, mean SCC noticeably increased from industrial farms toward traditional farms. The highest SCC was observed in semi-industrial farms when positive M-PCR results were observed for contagious pathogens, so that mean SCC had no significant difference between traditional farms and industrial ones.

Table 3 data revealed that prevalence of sub-clinical mastitis caused by contagious pathogens in the first, third and fifth parities were 34.1, 38.1 and 31.9%, respectively. *S. aureus* was more prevalent than *S. agalactiae* in each parity number. In the first parity the lowest mean SCC was for M-PCR⁻ status (124 000 cellsml⁻¹) whereas

increased for M-PCR⁺¹ (*S. agalactiae*; 312 000 cellsml⁻¹), M-PCR⁺¹ (*S. aureus*; 520 000 cellsml⁻¹), and then the highest mean SCC was for M-PCR⁺² (*S. aureus* + *S. agalactiae*; 1 280 000 cellsml⁻¹) status. In the third parity the highest mean SCC was for M-PCR⁺¹ (*S. aureus*; 653 000 cellsml⁻¹) and had significant difference with M-PCR⁺¹ (*S. agalactiae*; 478 000 cellsml⁻¹). In each row of Table 3 (with the exception of M-PCR⁻ status) parity number had significant effect on mean SCC.

DISCUSSION

Prevalence of contagious pathogens and the effect of pathogen type and diversity on mean SCC in different dairy cattle farms

The highest and the lowest prevalence of sub-clinical mastitis caused by contagious pathogens belong to traditional and industrial farms, respectively. As stated in

Table 2. Prevalence of contagious pathogens in different farm types and their effect on mean SCC in dairy cattle farm milk.

Pathogen diversity♣	Farm types♣♣						
	Industrial [†] (N=75)		Semi-Industrial ^{††} (N=105)		Traditional ^{†††} (N=86)		
	Prev. (%)	SCC×10 ³	Prev. (%)	SCC×10 ³	Prev. (%)	SCC×10 ³	
PCR ⁺¹	<i>S. aureus</i>	10.7	288 a [AB]	19.1	952 b [A]	19.8	565 a [A]
	<i>S. agalactiae</i>	2.7	200 a [B]	8.6	889 b [AB]	15.1	423 a [B]
PCR ⁺²	<i>S. aureus</i> + <i>S. agalactiae</i>	0	-	10.5	1760 a [AB]	9.3	588 a [B]
PCR ⁻	Contagious pathogen was not detected	40	61 a [B]	25.7	215 c [A]	25.6	455 a [A]

^{abc} Means within each column in each farm Type with different subscripts differ significantly (one-way ANOVA, $P < 0.05$). ^[ABC] Means within each row for each variable (PCR⁺¹, PCR⁺² and PCR⁻) with different superscripts differ significantly (one-way ANOVA, $P < 0.05$). ♣PCR⁻ (non major pathogen), PCR⁺¹ (one major pathogen) and PCR⁺² (two major pathogens were identified by M-PCR, simultaneously). ^{†††}Industrial farm (No. of cows in farm > 1000, hygienic milking system is good), semi-industrial farm (20 < no. of cows in farm ≤ 100, hygienic milking system is comparatively good), and traditional farm (no. of cows in farm ≤ 20, milking has been obtained with hand).

Table 3. Prevalence of contagious pathogens in different parity numbers and their effect on mean SCC in dairy cattle farm milk.

Pathogen diversity [†]	Parity numbers♣♣						
	1 (N=88)		3 (N=84)		5 (N=94)		
	Prev. (%)	SCC×10 ³	Prev. (%)	SCC×10 ³	Prev. (%)	SCC×10 ³	
PCR ⁺¹	<i>S. aureus</i>	17	520 b [CD]	22.6	653 a [BC]	16	987 ab [AB]
	<i>S. agalactiae</i>	9.1	312 b [CD]	15.5	478 b [BC]	7.4	1010 ab [AB]
PCR ⁺²	<i>S. aureus</i> + <i>S. agalactiae</i>	8	1280 a [A]	-	-	8.5	1390 a [A]
PCR ⁻	Contagious pathogen was not detected	42	124 b [A]	26.2	338 b [A]	32	207 c [A]

^{abc} Means within each column in each farm Type with different subscripts differ significantly (one-way ANOVA, $P < 0.05$). ^[ABC] Means within each row for each variable (PCR⁺¹, PCR⁺² and PCR⁻) with different superscripts differ significantly (one-way ANOVA, $P < 0.05$). [†]PCR⁻ (non major pathogen), PCR⁺¹ (one major pathogen) and PCR⁺² (two major pathogens were identified by M-PCR, simultaneously). ^{††}Animals were selected from amongst animals in the intermediate stage of lactation (75 to 100 days postpartum), which were not treated with antibiotics 7d prior to milk collection.

previous reports; common contagious pathogens have been reported to infect 7 to 40% of all cows (Fox and Gay, 1993; Sharif and Muhammad, 2009). In each farm type *S. aureus* was more prevalent than *S. agalactiae* which was in agreement with previous studies that is, *S. aureus* (26.92%) and *S. agalactiae* (8.33%) was isolated from sub-clinical bovine mastitis milk samples (Ali et al., 2011). These two pathogens were not simultaneously observed within industrial farms

whereas were significantly observed in semi-industrial and traditional farms. *S. aureus* was more prevalent than *S. agalactiae* within all of farm types that is, with performance of more hygienic treatments sub-clinical mastitis caused by contagious pathogens (*S. agalactiae*, particularly) have increasingly reduced. Because *S. agalactiae* respond well to antibiotic therapy and can be eradicated from dairy herds with good mastitis control practices (industrial farms)

whereas *S. aureus* infections remain the largest mastitis problem of dairy animals and cure rate with antibiotic therapy during lactation is very low (Khan and Khan, 2006). In industrial farms, pathogen diversity had no significant effect on mean SCC, that is, standard mastitis control and prevention practices recommended by national mastitis council of USA in industrial farms covered drastic effects of contagious pathogens on mean SCC, whereas pathogens diversity had significant

effect on mean SCC in semi-industrial farms so that the highest and the lowest SCC was observed in M-PCR⁺² and M-PCR⁻ status, respectively. In traditional farms pathogen diversity had no significant effect on mean SCC, because in Iran, traditional farms have Holstein-Golpayegani cross-breed dairy cows so that these animals have high resistance to infection and subsequent increased mean SCC. Table 2 data revealed that when M-PCR results were negative, mean SCC increased from industrial farms toward traditional ones, that is, the effect of farm type was significant probably for physical injury and/or mechanical milking reasons. As M-PCR results were positive the highest SCC was observed in semi-industrial farms, so that mean SCC had not significant difference between different farm types, that is, when M-PCR results would be positive the effect of farm type would be no significant. The lowest SCC was observed within industrial farms, then increased in traditional and semi-industrial farms, respectively (for M-PCR⁺¹ and then M-PCR⁺²). That due to good management and hygiene during maintenance and milking in industrial farms, as well as high resistance to infection in animals presented in Iranian traditional farms and have medium SCC with compared to animals presented in semi-industrial farms.

Prevalence of contagious pathogens and the effect of pathogen type and diversity on mean SCC in different dairy cattle parities

In this present study *S. aureus* was more prevalent than *S. agalactiae* in each parity number. Mean SCC between M-PCR⁻ and M-PCR⁺¹ status had no significant difference within the first parity, that is, the younger cows were more resistance to contagious pathogens. In the first parity the lowest and the highest mean SCC was for M-PCR⁻ and M-PCR⁺² (*S. aureus* + *S. agalactiae*), respectively. Our results emphasis the results of Rysanek et al. (2007). In the third parity the highest mean SCC was for M-PCR⁺¹ (*S. aureus*; 653 000 cellsml⁻¹) and had significant difference with mean SCC in M-PCR⁺¹ status (*S. agalactiae*; 478 000 cellsml⁻¹) so that mean SCC difference between M-PCR⁺¹ (*S. agalactiae*; 478 000 cellsml⁻¹) and M-PCR⁻ status (probably environmental pathogen existed; 338 000 cells ml⁻¹) was not significant, that is, the effect of *S. aureus* was more significant than *S. agalactiae* and the environmental pathogens. In the fifth parity significant difference was not observed in mean SCC between M-PCR⁺² and M-PCR⁺¹ status as well as between M-PCR⁺¹ (*S. aureus*) and M-PCR⁺¹ status (*S. agalactiae*). In each row of Table 3 (with the exception of M-PCR⁻) with increasing in parity number mean SCC significantly increased, that is, mean SCC had no significant difference between the first and the third parity or between the third and the fifth parity, but had significant difference between the first and the fifth parity, because in older cattle due to increased prevalence of infection and permanent glandular damage

from previous infections has significantly increased epithelial cell permeability and subsequent somatic cell entrance from blood to milk (Barlett et al., 1990; Sharif and Muhammad, 2008). As well as mastitis is characterized by an increase in milk barrier permeability and in the presence of a pathogen, macrophages release IL-8 and TNF- α , which results in PMN recruitment from blood to milk. This requires an increase in endothelial permeability, which induces not only the transfer of PMN from blood to milk but also other blood components (Marechal et al., 2011). In M-PCR⁻ status parity number had no significant effect on mean SCC, that is, Milk from uninfected quarters displays little change in SCC when number of lactations increase so that is in agreement with previous studies (Jones and Bailey, 2009). Therefore mean SCC was affected not only by pathogen type and diversity but also by parity number.

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