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PCR Assays for Detecting Major Pathogens of Mastitis in Milk Samples

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Abstract: Bovine milk samples were collected from cases of clinical and sub-clinical mastitis, respectively and examined bacteriologically and by simplex and multiplex PCR assays for detection of *Staphylococcus aureus, Escherichia coli* and *Streptococcus agalactiae*. *Escherichia coli* was the most common bacteria detected in the collected samples from clinical mastitis cases followed by *Staphylococcus aureus* and *Streptococcus agalactiae;* while *Staphylococcus aureus* was the most common bacteria followed by *Escherichia coli* and *Streptococcus agalactiae* in samples collected from sub-clinical mastitis cases. Compared with the cultural method, the simplex and multiplex PCR assays are less time consuming. It took less than 24 hours to be completed, while identification of bacteria to the species levels by conventional microbiological and biochemical methods required more than 72 hours. In conclusion, simplex and multiplex PCR assays can be used as a rapid, sensitive and specific routinely diagnostic tool to detect the presence of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* in milk samples.

Key words: Mastitis · *S. aureus* · *E. coli* · *S. agalactiae* · Culture · PCR

INTRODUCTION

Mastitis is the most common infectious disease affecting dairy cows. It is the most important cause of economic losses to the dairy industry in Egypt [1] and throughout the world [2]. The losses are due to reduced milk production, production of low quality milk, cost of drugs and veterinary services, increased culling rate and reduced reproductive efficiency [3].

The major cause of bovine mastitis is the infection of the udder by pathogenic bacteria. A wide variety of bacteria can be involved, but the most common mastitis pathogens in Egypt are *Staphylococcus spp. Escherichia coli* and *Streptococcus spp.* [4]. The pathogens responsible for the mastitis must be identified rapidly and accurately in order to monitor and control the infections in the dairy herd. Although conventional bacterial culture and biochemical tests could identify the microbial pathogens responsible for mastitis; they are time consuming, laborious and not highly specific [5].

Due to the limitations of cultural methods, the development of PCR-based methods provides a promising option for the rapid identification of bacteria. With these methods, identification of bacterial pathogens can be made in hours, rather than the days required for conventional cultural methods [6-9]. PCR can also improve the level of detection due to its high sensitivity. Theoretically, only a few cells of the pathogen are necessary to yield a positive diagnosis [10]. The presence of pathogens may be detected at earlier stages of infection and in carrier animals, when the numbers of bacteria in milk may be very low. The aim of this study was to use PCR assays for the detection of *Staphylococcus aureus*, *Escherichia coli and Streptococcus agalactiae* in mastitic milk samples.

MATERIALS AND METHODS

Samples: Two hundreds and 40 quarter milk samples were collected from Friesian cow (clinical and sub clinical mastitis cases) and mastitis free animals, respectively. Sampling of milk was performed according to Watts [11]. All collected samples were immediately kept in an insulated container with ice packs and transferred to the laboratory without delay to be objected to the milk scan instruments for estimation of somatic cell count (SCC), bacterial culturing and PCR assay.

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Estimation of Somatic Cell Count (SCC): The SCC was estimated in all samples using the somatic cell counter (Soma count 150 from BENTLY Company, Italy).

Bacteriological Culture: Milk samples were brought to room temperature and mixed thoroughly. Microorganisms causing mastitis were isolated and identified according to the methods previously reported by Carter [12] and Carter *et al*. [13].

DNA Extraction: Milk samples were mixed and a 300-µl sample added to 300 µl of lysis buffer (0.1M NaCl, 20 mM Tris-HCl, 1mM EDTA, 0.5% SDS and 100 µg of proteinase K/ml). After incubation at 37°C for 4 h. DNA was extracted and purified using phenol-chloroform method as described previously by Sambrook *et al*. [14]. The dried DNA pellet was dissolved in 50 µl of TE buffer (10 mM Tris HCl -1 mM EDTA, pH 7.8) and stored at -20°C until used.

Oligonucleotide Primers: Primers were synthesized, by (Fermentas, AB.Gene), (MWG, oligosynthesis - Germany). The sequences of different primers [9] included *Staphylococcus aureus* specific primer (Forward: 5` GGA CGA CAT TAG ACG AAT CA 3` and reverse: 5` CGG GCA CCT ATT TTC TAT CT 3`); *Escherichia coli* specific primer (Forward: 5` ATC AAC CGA GAT TCC CCC AGT 3` and reverse: 5` TCA CTA TCG GTC AGT CAG GAG 3`) and *Streptococcus agalactiae* specific primer (Forward: 5` CGC TGA GGT TTG GTG TTT ACA 3` and reverse: 5` CAC TCC TAC CAA CGT TCT TC 3`).

PCR Assays: Simplex PCR assay for amplification of each organism was performed using 25 µl PCR reaction volume. 12.5 µl of 2X PCR master mix (Fermentas, Germany), 150 ng of the DNA template, 0.5 µM of each primer and Up to 25 µl Nuclease free water were mixed in a PCR tube. The amplification was performed in a programmable heating block, (Primus Thermal Cycler, MWG Biotech, Germany). A total of 35 PCR cycles were run under the following conditions; denaturation at 94°C for 45 sec, annealing for 1 min (at 64 and 60°C for *Staphylococcus aureus* & *Escherichia coli* and *Streptococcus agalactiae*, respectively) and extension at 72°C for 2 min. After final cycle the preparations were kept for 10 min at 72°C to complete the reaction.

A one step multiplex PCR was developed using each of the primer sets previously used for the simplex PCR. 25 µl PCR reaction volume containing 12.5 µl of 2X PCR master mix (Fermentas, Germany), 0.5 mM $MgCl_2$, 250 ng of the DNA template, 25 pmol of each primer and Up to 25 µl Nuclease free water were mixed in a PCR tube. A total of 40 PCR cycles were run under the following conditions; denaturation at 94°C for 45 sec, annealing for 1 min at 62°C and extension at 72°C for 2 min. After final cycle the preparations were kept for 10 min at 72°C.

Amplification products were electrophoresed in 1.7% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a marker. Presence of 1318 bp, 232 bp and 405 bp DNA fragments indicated the presence of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* DNA, respectively.

RESULTS AND DISCUSSION

Prevention as a control measure of mastitis in dairy cattle needs sensitive, rapid and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. Conventional procedures for the identification of mastitis pathogens are labor-intensive and most of the commercial identification systems are not designed to identify important veterinary pathogens [15, 16]. The use of PCR detection and identification tests for mastitis pathogens that produced results in 1 day are specific, sensitive and cheap [8].

Out of 240 collected quarter milk samples, 124 mastitic milk samples were detected, 56 were from clinical cases and 68 were from sub clinical cases of bovine mastitis according to estimation of SCC of the fresh milk samples using the milk scan apparatus. Out of the 56 milk samples collected from clinical cases and examined bacteriologically; *Staphylococcus aureus, Escherichia coli and Streptococcus agalactiae* were recovered and identified in percentages of 16.1, 33.9 and 14.3%, respectively. Dual bacterial isolation [*Staphylococcus aureus* and *Escherichia coli* (7.1%); *Staphylococcus aureus* and *Streptococcus agalactiae* (7.1%); *Streptococcus agalactia*e and *Escherichia coli* (5.4%)] was also detected. Negative samples revealed 7.1% and 8.9% revealed mixed cultures of three or more environmental bacteria and accordingly were considered contaminated. On the other hand, the 68 milk samples from sub-clinical cases were examined bacteriologically. Bacterial isolates were identified as *Staphylococcus aureus* (29.4%), *Escherichia coli* (22.1%) and *Streptococcus agalactiae* (10.3%). Dual bacterial isolation [*Staphylococcus aureus* and *Escherichia coli* (8.8%); *Staphylococcus aureus* and *Streptococcus agalactiae*

Table 1: Detection of *S. aureus*, *E. coli* and *S. agalactiae* in 124 milk samples by culture, simplex PCR and multiplex PCR

	Test method											
	Culture				Simplex PCR				Multiplex PCR			
	Clinical mastitis		Sub-clinical mastitis		Clinical mastitis		Sub-clinical mastitis		Clinical mastitis		Sub-clinical mastitis	
Bacteria	N	%	N	%	N	%	N	%	N	%	N	%
S. aureus	9	16.1	20	29.4	10	17.9	23	33.8	10	17.9	21	30.9
E. coli	19	33.9	15	22.1	20	35.7	17	25.0	19	33.9	16	23.5
S. agalactiae	8	14.3	7	10.3	9	16.1	8	11.8	9	16.1	8	11.8
S. aureus+E. coli	4	7.1	6	8.8	4	7.1	6	8.8	4	7.1	6	8.8
S. aureus+S agalactiae	4	7.1	3	4.4	4	7.1	3	4.4	4	7.1	4	5.9
S. agalactiae+E. coli	3	5.4	3	4.4	3	5.4	3	4.4	3	5.4	3	4.4
No bacteria	4	7.1	8	11.8	1	1.8	2	2.9	2	3.6	5	7.4
Mixed 3 or more bacteria	5	8.9	6	8.8	5	8.9	6	8.8	5	8.9	5	7.4
Total	56	-	68	-	56	-	68	-	56	-	68	-

Table 2: Correlation between SCC / ml. and single bacterial infection in the examined milk samples

Bacterial infection	Minimum SCC / ml	Maximum SCC / ml	Mean	±SEM
Staphylococcus aureus	13000	200000	28420.6897	8835.53810
Escherichia coli	320000	320000	102805.8824	16195.05324
Streptococcus agalactiae	13000	17000	14500	284.52132

(4.4%); and *Streptococcus agalactiae* and *Escherichia coli* (4.4%)] was also detected. Also, 11.8 and 8.8% of samples revealed negative and mixed cultures of three or more environmental bacteria, respectively (Table 1).

The high prevalence of *Escherichia coli*-induced clinical mastitis encountered in the examined milk samples agrees with the findings of many investigators [17-19], who considered *Escherichia coli* organisms as major etiological agents of clinical mastitis, opportunistic environmental or enteric pathogens and the infected quarter could possibly serve as a reservoir for recurrent episodes of *Escherichia coli*-induced clinical mastitis [20, 21]. It is worth mentioning that cases of *Escherichia coli*-induced clinical mastitis are often of very short duration and milk samples may reveal negative cultures in approximately 20% of such cases [17, 22], accordingly, the culture-negative milk samples encountered in the present study may be attributed either to *Escherichia coli* or to other intra-mammary pathogens which could not be detected in the examined milk samples.

Staphylococcus aureus and *Streptococcus agalactiae*- induced clinical mastitis cases were also encountered in a high prevalence in the examined milk samples. This finding is similar to Bartlett *et al.* [23]; Dinsmore *et al.* [24] and Lafi *et al.* [25]. However, it disagrees with Hillerton *et al.* [26] and Sargeant *et al.* [27], who mentioned that these two bacteria are currently classified as causes of subclinical rather than clinical mastitis. This disagreement can be attributed to the poor mastitis control measures applied in some dairy farms. Nevertheless *Staphylococcus aureus* and *Streptococcus agalactiae* are considered as significant organisms associated with clinical and subclinical bovine mastitis worldwide [13, 28-30] due to persistent cow to cow spread, possibly via milking machines and perhaps by the hands of milkers [31]. Their main reservoirs are infected quarters [11, 32]. In addition, *Staphylococcus aureus* can also be isolated from the skin of the udder and teats and from many other sites in dairy cows as well as feed and caretakers [33, 34].

The inflammatory reaction caused by intra-mammary infection is most commonly measured as an increase in SCC. The samples which contained single infection with *Escherichia coli* recorded the highest mean SCC (102805.8824±16195.05324), in case of *Streptococcus agalactiae*, the samples recorded the lowest mean SCC (14500±284.52132), while the samples containing *Staphylococcus aureus* recorded an intermediate mean SCC (28420.6897±8835.5381) (Table 2). These results substantiate with Middleton *et al.* [35] and disagree with De Haas *et al.* [36].

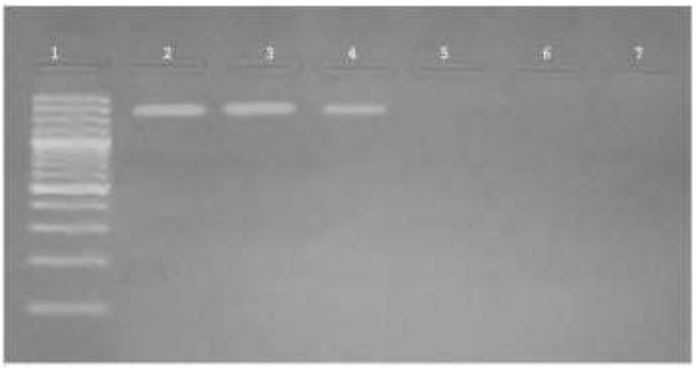

Fig. 1: PCR products (1318 bp) amplified from *Staphylococcus aureus* DNA. Lane 3, positive clinical mastitic milk sample; Lane 4, positive sub-clinical mastitic milk sample. Lane 5, negative clinical mastitic milk sample; Lane 6, negative sub-clinical mastitic milk sample. Lane 2, positive control; Lane 7, Negative control and Lane 1, 100 bp DNA marker.

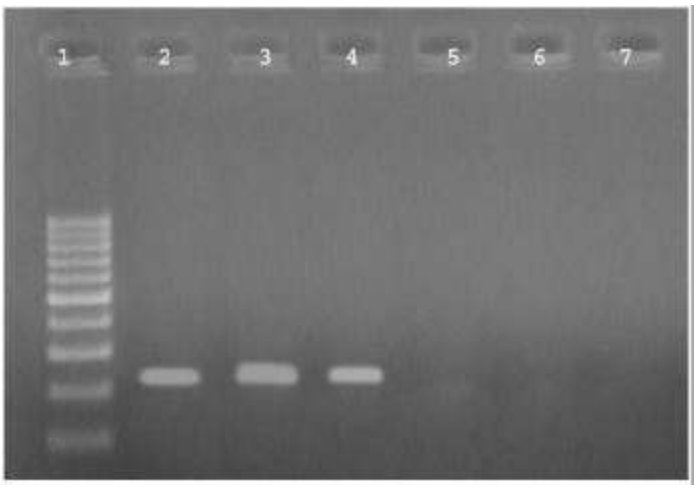

Fig. 2: PCR products (232 bp) amplified from *Escherichia coli* DNA. Lane 3, positive clinical mastitic milk sample; Lane 4, positive sub-clinical mastitic milk sample. Lane 5, negative clinical mastitic milk sample; Lane 6, negative sub-clinical mastitic milk sample. Lane 2, positive control; Lane 7, Negative control and Lane 1, 100 bp DNA marker.

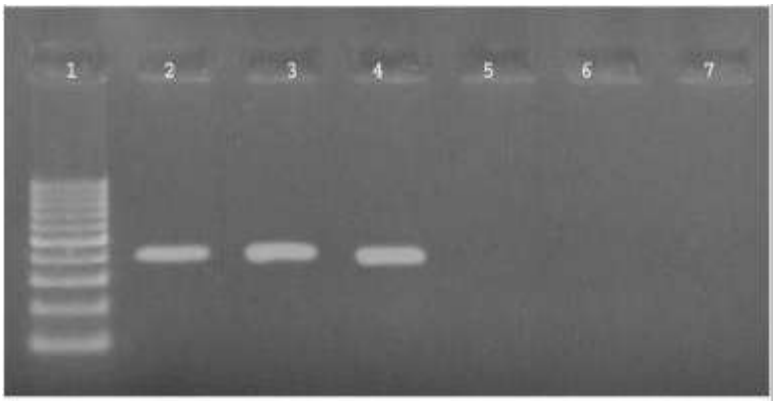

Fig. 3: PCR products (405 bp) amplified from *Streptococcus agalactia*e DNA from: Lane 3, positive clinical mastitic milk sample; Lane 4, positive sub-clinical mastitic milk sample. Lane 5, negative clinical mastitic milk sample; Lane 6, negative sub-clinical mastitic milk sample. Lane 2, positive control; Lane 7, Negative control and Lane 1,100 bp DNA marker.

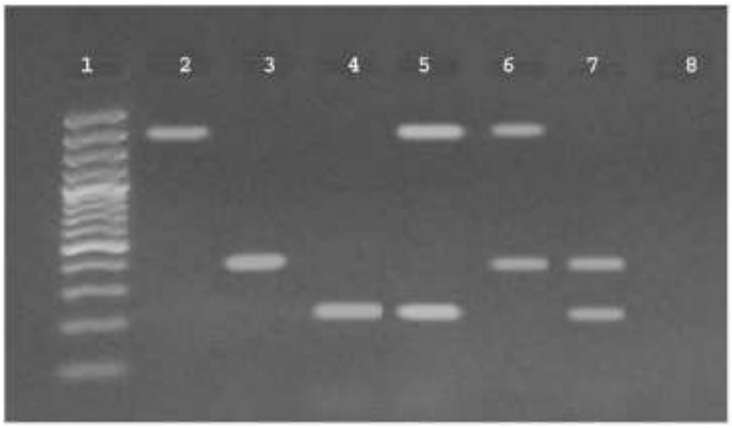

Fig. 4: Multiplex PCR products amplified from DNA. Lane 2, *Staphylococcus aureus*; Lane 3, *Streptococcus agalactiae*; Lane 4, *Escherichia coli*; Lane 5, *Staphylococcus aureus* and *Escherichia coli;* Lane 6*, Staphylococcus aureus* and *Streptococcus agalactiae*; Lane 7, *Streptococcus agalactiae* and *Escherichia coli.* Lane 1*,* 100 bp DNA marker and Lane 8, Negative control.

In the examined milk samples, the most common bacterial causes of bovine mastitis; *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* were detected by using single PCR assays. A multiplex PCR was used to allow the detection of multiple pathogens in a single reaction using smaller amounts of reagents and less time [5].

The number of mastitic milk samples positive for selected major pathogens of mastitis in the individual simplex PCR assays is listed in table 1. The reactivity of used primer sets with *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* in mastitic milk samples is shown in Figures 1, 2 and 3, respectively. The results indicated that, out of 56 milk samples from clinical cases of mastitis, 17.9, 35.7 and 16.1% of the samples were reacted positive when tested using *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* specific primer sets, respectively. On the other hand, out of 68 milk samples from sub-clinical cases of mastitis, 33.8%, 25.0% and 11.8% of the samples were reacted positive when tested using *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* specific primer sets, respectively. Dual bacterial detection [*Staphylococcus aureus* and *Escherichia coli* (7.1%); *Staphylococcus aureus* and *Streptococcus agalactiae* (7.1%); and *Streptococcus agalactiae* and *Escherichia coli* (5.4%)] were also indicated in samples from clinical mastitic cows and 8.8, 4.4 and 4.4%, respectively, in samples from sub-clinical mastitic cows. Also, 1.8 and 2.9% samples were negative in samples from clinical and sub-clinical mastitic cows, respectively (Table 1).

The data in table 1 and Figure 4 also showed the results of multiplex PCR using different primer sets of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae*. Out of 56 milk samples from clinical cases of mastitis, 17.9, 33.9 and 16.1% of the samples were reacted positive, respectively. On the other hand, out of 68 milk samples from sub-clinical cases of mastitis, 30.9, 23.5 and 11.8% of the samples were reacted positive, respectively. Dual bacterial detection [*Staphylococcus aureus* and *E. coli* (7.1%); *Staphylococcus aureus* and *Streptococcus agalactiae* (7.1%); and *Streptococcus agalactiae* and *Escherichia coli* (5.4%)] was also indicated in samples from clinical mastitic cows and 8.8, 5.9 and 4.4%, respectively, in samples from sub-clinical mastitic cows. Also, 3.6 and 7.4% samples revealed negative in samples from clinical and sub-clinical mastitic cows, respectively

The results also showed that, all the PCR test results were confirmed by culture tests. All examined milk samples from the sub clinical cases of bovine mastitis that didn't react with any primer set in PCR assays also gave negative result in culture tests, for the selected pathogens. It may be surmised that these mastitic milk samples might be free from any microbial infection although showed high SCC [37] or might be due to pathogens other than those selected for this study.

Compared with culture, the PCR assays are less time consuming. It takes less than 24 hours to complete, while identification of bacteria to the species levels by conventional microbiological and biochemical methods requires more than 72 hours. The results showed that the simplex and multiplex PCR were more sensitive than the

culture for detection of *Staphylococcus aureus, Escherichia coli* and *Streptococcus agalactiae* in milk. The same conclusion was reached by Riffon *et al*. [9] and Yamagishi *et al*. [38] and may be attributed to the fact that PCR detects living and dead organisms, since it is based on detection of organism DNA, while culture detects only living organisms. PCR assays could detect fewer numbers of organisms per milliliter of milk than could be detected by direct culture. It is not worthy to mention that all milk samples collected from mastitis free animals (40) were tested negative by PCR assays and culture method; a finding which indicated satisfactorily the specificity of culture and PCR assays.

One of the problems often encountered with multiplex PCR is a reduction in sensitivity than compared with simplex PCR. This may be due to the competition between individual reactions for dNTPs and *Taq* polymerase when multiplex primer sets are combined in a single reaction [39].

In conclusion, this study has shown that, as compared to the conventional culture method, the simplex and multiplex PCR assay can be used as a rapid and sensitive diagnostic tool to detect the presence of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* in milk samples.

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