

# Direct Identification of Major Pathogens of the Bubaline Subclinical Mastitis in Egypt using PCR

Khaled A. Abd El-Razik<sup>a,\*</sup>, Khaled A. Abdelrahman<sup>b</sup>, Youssef F. Ahmed<sup>a</sup>, Alaa M. Gomaa<sup>c</sup>, Hazem A. Eldebaky<sup>a</sup>

<sup>a</sup>Animal Reproduction Dept., <sup>b</sup>Parasitology Dept., National Research Center (NRC), Dokki, Giza, Egypt.

<sup>c</sup>Mastitis and Neonate Dept., Animal Reproduction Research Institute-Haram-Giza-Egypt.

\*[khaledemara707@yahoo.com](mailto:khaledemara707@yahoo.com)

**Abstract:** The present study aimed to evaluate the PCR assays (using Universal and specific primers) for detection of the major pathogens of bubaline subclinical mastitis directly from 160 buffaloes milk samples compared to bacterial examination. The bacteriological examinations showed incidence of 23.75%, 15.62%, 3.12% and 0.62% for *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysgalactiae* respectively. PCR was better in sensitivity and specificity than the conventional culture as it detected more positive results in culturally negative milk samples. The level of sensitivity achieved in our experiments ( $5 \times 10^1$  CFU/ml of milk) is applicable to milk sample analysis without sample enrichment. The results suggest that PCR assay especially the one working with universal primers could be used as an alternative method in routine diagnosis for rapid, sensitive, and specific simultaneous detection of *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysgalactiae* in milk samples. [Journal of American Science 2010; 6(10):652-660]. (ISSN: 1545-1003).

**Key words:** Buffaloes, subclinical mastitis, Staph, Strept, E.coli, PCR.

## 1. Introduction:

Buffaloes are the main dairy animals in some developing countries worldwide despite this species tends to have relatively slow rate of reproduction and more reproductive problems such as inactive ovaries, long calving intervals and mastitis (Hussein, 2002; Piccinini et al., 2006).

Subclinical mastitis is a major problem affecting dairy animals all over the world. It causes enormous losses for breeders and consequently influences the national income of the country (McDougall et al., 2009). Economic losses are due to (a) loss in milk production, (b) discarding abnormal milk and milk withheld from cows treated with antibiotics, (c) degrading of milk quality and price due to high bacterial or somatic cell count (SCC), (d) costs of drugs, (e) veterinary services and increased labor costs, (f) increased risk of subsequent mastitis, (g) herd replacement, and (h) problems related to antibiotics residues in milk and its products (Bramely et al., 1996; Getahun et al., 2008).

Health status of mammary gland in milking animals contributes greatly in the economic importance of the farm animals. Despite susceptibility to mastitis is low in buffaloes when compared to cattle (Saleh, 2005), the poor management conditions practiced by small buffalo

holders in rural areas may anticipate in increased percentage of subclinical mastitis.

Subclinical mastitis causes reduction in milk quality and its market value as well as it is responsible for up to 70% of the losses in mastitis (Kirk and Bartlett, 1988). Moreover, quarter-wise prevalence of intramammary infection in buffalo was 66%, especially during the periparturient period, whereas the incidence is highest during the 30 days after calving (Moroni et al., 2006).

Subclinically infected cows are intermittent shedders of organisms and may cycle through low and high shedding patterns during lactation. Milk culture may yield no bacteria from truly subclinically infected glands due to the presence of very low numbers of pathogens when samples are collected (Cai et al., 2003). Negative cultures may also be due to bacteria inhibited by residual therapeutic antimicrobials or leukocytes. Environmental contaminants and intracisternal microorganisms can also represent a major problem in the interpretation of culture results. Moreover, microbiological culture of milk is time consuming. Species identification by standard biochemical methods requires more than 48 h to complete (Phuektes et al., 2001).

Due to the limitations of conventional culture, simplex and multiplex PCR protocols have been developed for identification of the various

mastitis pathogens (Daly et al., 2002; Meiri-Bendek et al., 2002; Anand Kumar, 2009; Kozyska et al., 2010). These PCR methods offer the option of identification of bacteria within hours. PCR can also improve the level of detection because of its ability to detect low numbers of organisms. Mastitis pathogens could probably be detected in carrier animals or at earlier stages of infection in clinical cases. PCR also has the potential to be extremely specific and to discriminate between closely related microorganisms, such as *S. parauberis* and *S. uberis* (Jayarao et al., 1991).

The aim of this study was to improve direct diagnosis of subclinical mastitis in buffaloes by simple and specific method, a Polymerase Chain Reaction (PCR) assay. PCR was developed using universal and specific primers for simultaneous detection of the four major bacterial causes of bovine mastitis, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*.

## 2. Material and Methods:

The current work was carried out on dairy buffaloes reared by local farmers at villages of different Egyptian governorates (Menufia and Kalubia) during the period between 2009-2010.

### Milk Samples

A total of 160 forequarter milk samples from 40 buffaloes with subclinical mastitis based on the absence of visible abnormalities of milk secretions and positive California Mastitis Test (CMT) were used in this study.

The examined udders were thoroughly washed, dried with a clean towel and the teats were sprayed with 70% ethanol. After that the first few jets of milk were discarded and 50 ml of milk samples from each quarter were collected in a sterile McCartney bottle (Blood and Henderson, 1986). All samples were tested by the CMT, and then kept at 4 °C and transported immediately to the laboratory. Each sample was divided into two parts, each in a sterile McCartney bottles. One was incubated at 24 h for bacteriological examination and the other part was stored in a freezer (at approximately -20 °C) for PCR testing.

### California Mastitis test

California mastitis test (CMT) was carried out according to Schalm and Noorlander (1957). According to the changes of colour and grade of gel formation, results were interpreted as negative, trace, 1+, 2+, and 3+, as described by Schalm et al. (1971).

### Bacteriological Examination

Milk samples were incubated aerobically at 37°C for 24 h then centrifuged at 3000 rpm for 20 minutes. The supernatant fluid was discarded and a loopful from the sediment was streaked onto the surface of mannitol salt agar, blood agar and MacConkey agar plates. The plates were incubated at 37°C for 24-48 h, and examined for bacterial growth. The isolated colonies were identified according to Finegold and Baron (1986).

### Polymerase Chain Reaction

#### 1- Bacterial Strains and Growth conditions.

The organisms used in this study include *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysgalactiae* isolated from bovine mastitis which were kindly offered by the dairy unit of the Institute of Animal Reproduction and identified biochemically by the API Staph and Strep systems accordingly. All organisms were cultured in TSB (Difco Laboratories, Detroit, Mich.) at 37°C for about 17 h before DNA extraction. Cell numbers were determined by the preparation of serial dilutions of an overnight culture in phosphate-buffered saline (PBS) and plating on blood agar (Columbia agar base supplemented with 5% defibrinated sheep blood).

#### 2-Preparation of Bacterial DNA for PCR Using the Dneasy Blood & Tissue Kit (Qiagen Co., Cat. no. 69504) with Some Modifications

Bacteria were grown overnight in 20 ml of TSB, and then 1.5 ml of this culture was centrifuged at 5,000 Xg for 10 min. then heat in heat block at 95°C for 10 min then put on ice The Dneasy Tissue kit from Qiagen was used by following the manufacturer's recommendation.

#### 3-Phenol-Chloroform Extraction of DNA from Milk Samples

Frozen milk was thawed at room temperature and 500 µl of sample were mixed with 199 µl of NET buffer and 100 µl of 24% sodium dodecyl sulphate (SDS). After incubation at 80°C for 10 minutes, the mixture was cooled on ice. Proteinase K was added to the mixture at a concentration of (325 mg/ml), then the mixture was incubated at 50°C for 1.5 hours, followed by treatment with phenol: chloroform: isoamyl alcohol (25:24:1) as reported by Romero and Lopez-Goni (1999). The pellet was dried and dissolved in 25 µl sterile distilled water and stored at -20°C.

#### 4-PCR Primers

PCR primers were designed from highly divergent and species specific regions of the DNA coding for 16S and 23S rRNA based on previously

published sequence entries available in the Gene Bank database (*E. coli*, GI no. 42756; *S. aureus*, GI no. 288516; *S. agalactiae*, GI no. 2353759; *S. dysgalactiae*, GI no. 560494). Genes encoding rRNA were used as target sequences rather than genes encoding mRNA because of signal enhancement due

to presence of several copies of genes encoding rRNA in the genome (Riffon *et al.*, 2001). The sequences, specificities, and G+C contents are summarized in Table 1. The primer combinations, the annealing temperatures, and the lengths of the amplified products are summarized in Table 2.

**TABLE 1. Oligonucleotide primer sequences used for PCR**

Primer	Specificity	Sequence (5'-3')	G+C Content (%)
Uni 678	Universal	AGT GGA ATT CCA TGT GTA GC	45
Uni 888	Universal	GAG TGC TTA ATG CGT TAG CT	45
Eco 2083	<i>E. coli</i>	GCT TGA CAC TGA ACA TTG AG	45
Eco 2745	<i>E. coli</i>	GCA CTT ATC TCT TCC GCA TT	45
Sau 327	<i>S. aureus</i>	GGA CGA CAT TAG ACG AAT CA	45
Sau 1645	<i>S. aureus</i>	CGG GCA CCT ATT TTC TAT CT	45
Sag 40	<i>S. agalactiae</i>	CGC TGA GGT TTG GTG TTT ACA	48
Sag 445	<i>S. agalactiae</i>	CAC TCC TAC CAA CGT TCT TC	50
Sdy 105	<i>S. dysgalactiae</i>	AAA GGT GCA ACT GCA TCA CTA	43
Sdy 386	<i>S. dysgalactiae</i>	GTC ACA TGG TGG ATT TTC CA	45

**TABLE 2. Primer Conditions During PCR**

Forward Primer	Reverse Primer	Annealing Temp (°C)	Size of Product Amplified (bp)
Uni 678	Uni 888	56	210
Eco 2083	Eco 2745	57	662
Sau 327	Sau 1645	64	1,318
Sag 40	Sag 445	60	405
Sdy 105	Sdy 386	57	281

5- PCR Amplification With Modification of the Method Reported by Riffon *et al.* (2001).

All reactions were carried out in a final volume of 50 µl. Volumes of 200 ng of extracted DNA template or 5 µl of bacterial preparation, 5 µM primer, 25 µl of Taq PCR Master Mix (Taq PCR Master Mix Kit, Cat no 201443, Qiagen®), were added to a 0.5 ml microcentrifuge tube. A pre-PCR step at 95°C for 2 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45s, annealing (at the temperature in Table 2) for 45s, and extension at 72°C for 45s. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were analyzed by electrophoresis on 1.5% agarose gel.

#### 6-Sensitivity Test

This work concentrated on the sensitivity of the universal primers (Uni 678 and Uni 888) for future use in diagnosis of mastitis on tank milk. We

used *S. aureus* as a model for testing. Tenfold serial dilution was made from the inoculated milk samples using uninoculated pasteurized milk as a diluent. Parallel dilutions of *S. aureus* were also made in distilled water. These dilutions were then plated on sheep blood agar and after incubation numbers of bacterial colonies were counted. DNA was extracted by the previously mentioned method and the sensitivity of PCR was then compared with dilutions containing organisms at  $5 \times 10^{10}$  cfu/ml to  $5 \times 10^1$  cfu/ml using the PCR conditions described above.

#### 3. Results:

Isolation and Identification of the Isolated Organisms.

The bacteriological examination of milk samples collected from sub-mastitic buffaloes shown in Table (3) reported an incidence of 23.75% for *E. coli* infection followed by *S. aureus* (15.62%), *S. agalactiae* (3.12%) and *S. dysgalactiae* (0.62%).

**TABLE 3. Screening of Mastitic Milk Samples for Selected Pathogens of Bubaline Mastitis by PCR Test**

Bubaline Mastitis Pathogens	No.&% of Mastitic Milk Positive in Culture Test	No.&% of Mastitic Milk Positive in Uni-PCR Test	No.&% of Mastitic Milk Positive in Sp-PCR Test
<i>E. coli</i>	38 (23.75%)	38 (23.75%)	37 (23.12%)
<i>S. aureus</i>	25 (15.62%)	25 (15.62%)	23 (14.37%)
<i>S. agalactiae</i>	5 (3.12%)	5 (3.12%)	5 (3.12%)
<i>S. dysgalactiae</i>	1 (0.62%)	1 (0.62%)	1 (0.6%)

The results of Uni-PCR (using Uni 678 and Uni 888 primers) shown in Table 3 were identical to that of the bacterial isolation (23.75%, 15.60%, 3.12% and 0.62%). This means that the Uni-PCR was as sensitive as the bacterial isolation.

Using the specific primers, the results of Sp-PCR were less in sensitivity than bacterial isolation and Uni-PCR concerning *E. coli* and *S. aureus* only (23.12% and 14.37% respectively).

Table 4 showed that the PCR using universal primers in culturally positive milk samples was as

sensitive as the bacterial isolation, while the PCR using specific primers was less sensitive than both the bacterial examination and Uni-PCR.

Also using Uni-PCR there were positive results in culturally negative milk samples in all the previous bacterial strains especially in *S. aureus* but not in *S. agalactiae*.

On the other hand the same was noticed using the Sp-PCR but with less extent and only for *E. coli* and *S. aureus* as shown in Table 4.

**TABLE 4. Comparison of Detection of *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysgalactiae* in Milk Samples by Culture, Uni-PCR and Sp-PCR.**

Organism	Culture	Uni-PCR		Sp-PCR		Total
		(+)	(-)	(+)	(-)	
<i>E. coli</i>	(+)	38	0	37	1	38
	(-)	2	120	3	119	122
	Total	40	120	40	120	160
<i>S. aureus</i>	(+)	25	0	23	22	5
	(-)	8	127	3	132	135
	Total	33	127	26	134	160
<i>S. agalactiae</i>	(+)	5	0	5	0	5
	(-)	0	155	0	155	155
	Total	5	155	5	155	160
<i>S. dysgalactiae</i>	(+)	1	0	1	0	1
	(-)	1	158	0	159	159
	Total	2	158	1	159	160

Uni-PCR: PCR using universal primers, Sp-PCR: PCR using specific primers



Fig.1

Fig. 1. PCR amplification (210 bp) of purified bacterial DNA by using universal primers. Lanes: (A), *E. coli*; (B), 100 bp DNA ladder; (C), negative control without DNA; (D), *S. aureus*; (E), *S. agalactiae*, (F), *S. dysgalactiae*.

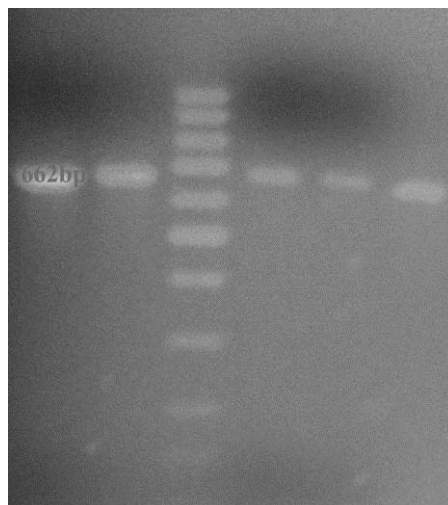


Fig.2

Fig. 2. Agarose gel showing amplification products (662 bp) with *E.coli* using the Eco 2083 and Eco 2745 primer pairs. Lanes (A&B), *E. coli* DNA only; Lane (C), 100 bp DNA ladder; Lanes (D, E and F), DNA extracted from *E. coli* in milk.

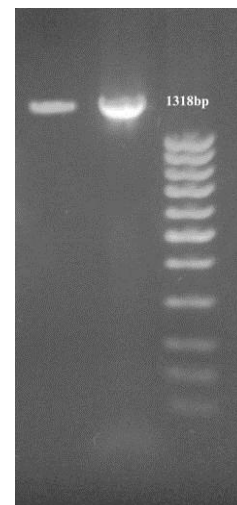


Fig.3

Fig. 3. Agarose gel showing amplification products (1,318 bp) with *S. aureus*, using the Sau 327 and Sau 1645 primer pairs. Lane (A), DNA extracted from *S. aureus* in milk; Lane (B), *S. aureus* DNA only; Lane (C), 100 bp DNA ladder.

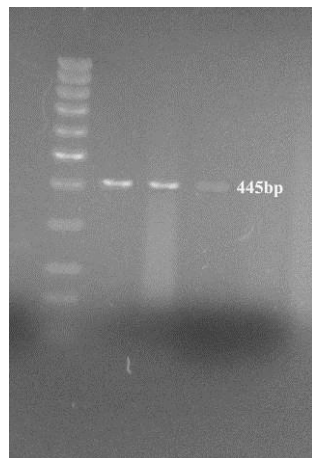


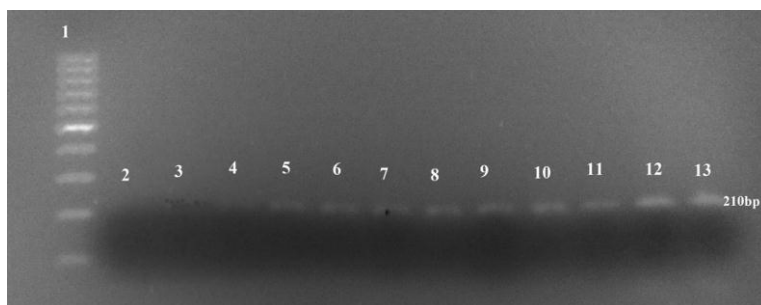
Fig.4

Fig. 4. Agarose gel showing amplification products (405 bp) with *S. agalactiae*, using the Sag 40 and Sag 445 primer pairs. Lane (A),100-bp DNA ladder; Lane (B), *S. agalactiae* DNA only, Lane (C&D), DNA extracted from *S. agalactiae* in milk; Lane (E), negative control.



Fig.5

Fig. 5. Agarose gel showing amplification products (281 bp) with *S. dysgalactiae*, using the Sdy 105 and Sdy 386 primer pairs. Lane (A),100 bp DNA ladder; Lane (B), *S. agalactiae* DNA only, Lane (C), DNA extracted from *S. dysgalactiae* in milk



**Fig.6**

**Fig.6. Sensitivity of PCR assay in detecting DNA from milk spiked with *S. aureus*. Universal Primers Uni 678 and Uni 888 were used. Lane 1: 100 bp ladder, Lanes 2&3: negative control, Lanes 4 to lanes 13: amount of bacteria used ( $5 \times 10^1$  to  $5 \times 10^{10}$ )**

The Uni 678 and Uni 888 primer set reacted with *S. aureus* spiked milk samples from  $5 \times 10^{10}$  CFU/ml to  $5 \times 10^1$  CFU/ml and the intensity of PCR products were good. The intensity of the PCR product was very faint at  $5 \times 10^1$  CFU/ml (Fig.6).

#### 4. Discussion:

Subclinical mastitis is the most serious type of disease as the infected animal shows no obvious symptoms and secretes apparently normal milk for a long time, during which causative organisms spread infection in the herd, so it is an important feature of the epidemiology of many forms of bovine mastitis (Bakken and Gudding, 1982).

The present study aimed to screen the subclinical form of mastitis as it is considered 15-40 times more prevalent than clinical form and accounts for greater losses in terms of milk production (Harmon *et al.*, 1994) and represents a reservoir of infectious organisms, besides it is related to approximately 70% of economic losses of mastitis (Varshney and Naresh, 2004). Moreover, subclinical mastitis is 3-40 times more prevalent than its clinical counterpart and causes greater economic losses than clinical mastitis (Almaw *et al.* 2008; George *et al.* 2008).

Because mastitis is one of the most costly and troublesome diseases in dairy cows in Egypt (Seleim *et al.*, 2002), its vital importance in its association with many zoonotic diseases in which milk acts as a vehicle of pathogens causing tuberculosis and brucellosis (Shoshani *et al.*, 2000) and because in its subclinical form a major part of the cow's udder has already been affected and the quality and quantity of milk reduced (Harmon *et al.*, 1994), therefore its detection in its subclinical form is very important.

Early diagnosis of mastitis is a must for reduction of production losses and for enhancing the prospects of recovery. Also, the identification of sub

clinically infected gland is urgently required for successful control of mastitis in dairy animals (Ahmed *et al.*, 2008)

The present study was designed to investigate subclinical mastitis in the main Egyptian dairy animals which are buffaloes that produce 65% of dairy product with special concept to find a practical marker for its early diagnosis.

The CMT is a reliable, easy, rapid and cheap tool helping in diagnosis and controlling the disease as it directs attention to individual mammary quarter that is secreting milk of high somatic cell content (SCC) (Leslie *et al.*, 2002; Abdel-Rady and Sayed, 2009). Moreover, Park *et al.* (1982) reported that CMT was in a good agreement with bacteriological results.

Because quantification of SCC is not possible in many countries in which buffalo farming is developing, the simple CMT may be a practical alternative as reported by Bhindwale *et al.* (1992) and Dhakal (1994). Therefore in this study a total of 160 quarter foremilk samples (QFS) from 40 buffaloes with subclinical mastitis based on the absence of visible abnormalities of milk secretions and positive CMT were analyzed.

From table (3) we can find that the incidence of subclinical mastitis in buffaloes depending on the bacterial cultivation was the highest in *E. coli* infection (23.75%), *S. aureus* (15.62%) followed by, *S. agalactiae* (3.12%) and *S. dysgalactiae* (0.62%). This comes in agreement with El-Khodery and Osman (2008) who reported that the bacteriological examination of buffaloes milk samples with acute mastitis revealed that coliform bacteria was the most common pathogen followed by *S. aureus* then *S. uberis*, and *S. agalactiae*.

This also came to some extent with Ahmed *et al.* (2008) who reported high incidence of bacteria isolated from milk samples of Egyptian buffalo-cows suffering from sub-clinical mastitis where the most

prevalent isolates were *E. coli* (94.99%), *S. epidermidis* (78.33%), *C. bovis* (55%), *Klebsiella* spp. (51.67%), *S. uberis* (46/67%), *S. aureus* (33.33%) and *S. agalactiae* (31.67%).

Our study revealed high incidence of *E. coli* infection in Egyptian buffaloes compared with its incidence in previous reports of subclinical mastitis in the Egyptian cattle (16.25%) as reported by *Abdel-Rady and Sayed*, (2009), this may be due to the presence of some characteristics in buffaloes that may contribute to greater risk of mastitis such as more pendulous udder and longer teats. Besides, *Krishnaswamy et al.* (1965) stated that teat sphincter of buffaloes have smoother muscular fiber in such a way it constitutes a better barrier to microorganism invasion than cow's teat sphincter. This is logic with the hygienic measures concerning buffaloes with small holders in Egypt.

Although PCR assays were earlier developed to detect the major pathogens of mastitis in cow's milk (*Riffon et al. 2001; Phuektes et al. 2001*), the processing of mastitic milk samples was not simplified and also the PCR assays were not adopted for identification of the mastitis pathogens of buffaloes. Therefore this study aimed to evaluate the PCR test for detecting the major pathogens of bovine mastitis (BM) directly from the mastitic milk samples of buffaloes, with simple and inexpensive method of sample processing.

Specific primers described here (Table 1 and Fig. 1-5) were proven to be specific since on agarose gel only one band was observed for each set of primers and no signal was detected with negative controls.

The primers for *E. coli*, *S. aureus* were designed based on a DNA sequence coding for 23S rRNA, while primers for *S. agalactiae* and *S. dysgalactiae* were based on DNA coding for 16S rRNA. All the signals were very obvious. The difference of signal cannot be explained by the amount of DNA used for PCR because the same amount (200 ng) was used for all bacteria. Therefore, it is possible that the difference in the copy numbers of the coding regions for these particular probes could explain the phenomenon (*Bentley and Leigh, 1995*).

The universal primer sets used as positive controls, Uni 678 plus Uni 888 were designed from DNA regions coding for 16S and 23S rRNA, respectively. Even though the nucleotide sequence data comparison (BLASTN 2.0) showed that the two universal primers are conserved in all pathogens of BM, the sequences can vary by 1 or 2 nucleotides, therefore varying the signal intensity.

When PCR with universal primers was used to detect pathogens directly in milk samples as shown

in table 4, it was as sensitive as the bacterial isolation but it was less sensitive than conventional culture when used with the specific primers (only for *E. coli* and *S. aureus*). These findings suggest that PCR inhibition was the problem encountered when PCR was used on milk samples (*Higuchi, 1989; Toye et al., 1998*). PCR-inhibiting substances may remain in the samples despite the use of DNA purification methods.

In subclinical mastitis, samples with no bacterial growth are generally very common; in various studies on subclinical mastitis, the percentage of culture-negative samples ranged from 28.7% to 38.6% as reported by *Koivula et al.* (2007) and *Bradley et al.* (2007) respectively.

The ability of PCR using either universal or specific primers to detect positive reactors in culturally negative milk samples shown in table 4, may be due to the inability of bacteria to grow even after 48 h of conventional culture in more than 30% of milk samples from clinical and subclinical bovine mastitis. The "no-growth" samples are problematic for mastitis laboratories, veterinarians, and dairy producers as reported by *Taponen et al.* (2009). Presence of many antibacterial substances in milk such as lactoferrin, lysozyme, lactoperoxidase, components of complement, and immunoglobulins may be another reason (*Rainardand Riollet, 2006*). Their concentrations remain at a low level in healthy quarters, but substantially increase during mastitis. It can be hypothesized that these substances operating in synergy with milk leukocytes could contribute to death of bacteria in mastitic milk (*Taponen et al., 2009*).

Compared with culture, this PCR is less time consuming. It takes less than 24 h to complete, while identification of bacteria to the species levels by conventional microbiological and biochemical methods requires more than 48 h.

In the aim to evaluate the sensitivity level of PCR test and to reduce its cost and duration, a study to compare the sensitivities of the test with universal primers Uni 678 and Uni 888 (using sterile milk samples spiked with different concentrations of *S. aureus* from  $5 \times 10^{10}$  CFU/ml of milk till  $5 \times 10^1$  CFU/ml) was performed. This Uni-PCR succeeded to detect up to  $5 \times 10^1$  CFU of *S. aureus*/ml of milk. This result is better than that of *Riffon et al.* (2001) who indicated a detection limit between  $3.12 \times 10^2$ -  $1.25 \times 10^3$  CFU/ml of milk, also was better than that of *Cremonesi et al.* (2006) and *Anand Kumar* (2009) who reported detection limit of  $7 \times 10^1$  CFU/ml and  $5.5 \times 10^2$  CFU/ml respectively. The detection limit of Uni- PCR was sensitive enough to be used as diagnostic tool for direct identification of the pathogens in buffaloes milk.

In conclusion, a simple and rapid PCR-based assay for the detection of the major pathogens involved in BM is described here. The test, directly performed from milk samples without a culture step, is specific for *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysgalactiae*. The Uni-PCR is suitable for further studies on tank milk.

#### Corresponding author

Khaled A. Abd El-Razik  
Animal Reproduction Dept  
National Research Center (NRC), Dokki, Giza, Egypt  
[khaledemara707@yahoo.com](mailto:khaledemara707@yahoo.com)

#### 5. References:

1. Abdel-Rady A, Sayed M (2009) Epidemiological studies on subclinical mastitis in dairy cows in Assiut Governorate. *Veterinary World* 2:373-380.
2. Ahmed WM, Sherein I, Ghada M Nabil (2008) Observations on sub-clinical mastitis in buffalo-cows with emphasis on measuring of milk electrical resistance for its early detection. *Global Veterinaria* 2:41-45.
3. Almaw G, Zerihun A, Asfaw Y (2008) Bovine mastitis and its association with selected risk factors in small holder dairy farms in and around Bahir Dar, Ethiopia. *Trop Anim Health Prod* 40: 427-432.
4. Anand Kumar P (2009) Evaluation of PCR test for detecting major pathogens of bubaline mastitis directly from mastitic milk samples of buffaloes. *Trop Anim Health Prod* 41:1643-1651.
5. Bakken G, Gudding R (1982) The interdependence between clinical and subclinical mastitis. *Acta Agri.Scandin* 32:17.
6. Bentley RW, Leigh J A (1995) Determination of 16S ribosomal RNA gene copy number in *Streptococcus uberis*, *S. agalactiae*, *S. dysgalactiae* and *S. parauberis*. *FEMS Immunol. Med. Microbiol* 12:1-7.
7. Bhindwale S, Supekar PG, Shukla PC (1992) Subclinical mastitis detection in buffaloes by California Mastitis Test (CMT). *Livest Adviser* 17:14-15.
8. Blood DC, Henderson JA (1986) Veterinary medicine, 3<sup>rd</sup> ed., London: *BaillierTindall and Gassel*.
9. Bradley AJ, Leach KA, Breen JE, Green LE, Green MJ (2007) Survey of the incidence and etiology of mastitis on dairy farms in England and Wales. *Vet Rec*160: 253-258.
10. Bramley AJ (1996) Current concepts of bovine mastitis. 4<sup>th</sup> Ed. National Mastitis Council, Inc. Arlington, VA.
11. Cai HY, Archambault M, Gyles CL, Prescott JF (2003) Molecular genetics methods in the veterinary clinical bacteriology laboratory: current usage and future applications. *Animal Health Research Reviews* 4: 73-93.
12. Cremonesi P, Castiglioni B, Malferrari G, Biunno I, Vimercati C, MoroniP, Morandi S, Luzzana M (2006) *Technical Note*: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. *J. Dairy Sci* 89:163-169.
13. Daly P, Collier T, Doyle S (2002) PCR-ELISA detection of *Escherichia coli* in milk. *Letters in Applied Microbiology* 34:222-226.
14. Dhakal IP (2006) Normal somatic cell count and subclinical mastitis in murrh buffaloes. *J.Vet. Med. B* 53: 81-86.
15. El-Khodery SA, Osman SA (2008) Acute coliform mastitis in buffaloes (*Bubalus bubalis*): clinical findings and treatment outcomes. *Trop Anim Health Prod* 40:93-9.
16. Finegold SM, Baron EJ (1986) "Diagnostic Microbiology" 7<sup>th</sup> Edition. The C.V. Mosby Co. St. Louis, London.
17. George LW, Divers TJ, Ducharme N, Welcome FL (2008) *Rebhun's Diseases of Dairy Cattle* (Saunders Elsevier, St Louis, USA).
18. Getahun K, Kelay B, Bekana M, Lobago F (2008) Bovine mastitis and antibiotic resistance patterns in Selalle small holder dairy farms, central Ethiopia. *Trop Anim Health Prod* 40: 261-168.
19. Harmon RJ, Clark TW, Trammell DS, Smith BA, Torre PM, Hemken RW (1994) Influence of copper status in heifers on response to intramammary challenge with *Escherichia coli* endotoxin. *J. Dairy Sci.* 77 (Suppl. 1), 198.
20. Higuchi R (1989) Simple and rapid preparation of samples for PCR. Pages 31-38 in PCR technology: Principles and Applications for DNA amplification. H. A. Ehrlich, ed. Stock Press, New York, NY.
21. Hussein FM (2002) Reproduction in the buffalo-cow-An overview. Proceeding of the annual 14<sup>th</sup> congress of Egyptian Society of Animal Reproduction and Fertility, Giza, pp: 5.
22. Jayarao BM, Dore JJ, Baumbach GA, Matthews KR, Oliver SP (1991) Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. *J. Clin. Microbiol.* 29:2774-2778.
23. Kirk J, Bartlett (1988) Economic impact of mastitis in Michigan Holstein herds using a



- computer records programme. *Agri-Practice* 9: 3-6.
24. Koivula M, Pitkala A, Pyorala S, Mantysaari E (2007) Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. *Acta Agric.Scand.A* 57:89–96.
25. Kozytska S, Staul D, Pawlik MC, Hensen S, Eckart M, Ziebuhr W, Witte W, Ohlsen K (2010) Identification of specific genes in *Staphylococcus aureus* strains associated with bovine mastitis. *Veterinary Microbiology* (under publication).
26. Krishnaswamy S, Vedanayaham R, Varma K (1965) Studies on mastitis in cattle. *Indian Veterinary Journal* 42: 92-103.
27. Leslie KE, Jansen JT, Lim GH (2002) Opportunities and implications for improved on-farm cow side diagnostics. *Proc De Laval Hygiene Symp* 147-160.
28. McDougall S, Parker KI, Heuer C, Compton CWR (2009) Compton A review of prevention and control of heifer mastitis via non-antibiotic strategies. *Veterinary Microbiology* 134: 177–185.
29. Meiri-Bendek I, Lipkin E, Friedmann A, Leitner G, Saran A, Friedman S, Kashi, Y (2002) A PCR-based method for the detection of *Streptococcus agalactiae* in milk. *J. Dairy Sci* 85: 1717–1723.
30. Moroni P, Rossi CS, Pisoni G, Bronzo V, Castiglioni B, Boettcher PJ (2006) Relationship between somatic cell count and intramammary infection in buffaloes. *J. Dairy Sci.* 89: 998–1003.
31. Park JH, Kim KH, Ani SH (1982) Efficiency of C.M.T. for diagnosis of bovine mastitis. *Korean J Vet Res* 22:237.
32. Phuektes P, Mansell PD, Browning GF (2001) Multiplex Polymerase Chain Reaction Assay for Simultaneous Detection of *Staphylococcus aureus* and Streptococcal Causes of Bovine Mastitis. *J. Dairy Sci.* 84:1140–1148.
33. Piccinini R, Miarelli M, Ferri B, Tripaldi C, Belotti M, Dapra V, Orlandini S, Zeconi A (2006) Relationship between cellular and whey components in buffalo milk. *J. Dairy Res* 73: 129-133.
34. Rainard P, Riollot C (2006) Innate immunity of the bovine mammary gland. *Vet Res* 37: 369–400
35. Riffon R, Sayasith K, Khalil H, Dubreuil P, Drolet M, Lagace J (2001) Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *J. Clin. Microbiol.*7: 2584–2589.
36. Romero C, Lopez-Goni I (1999) Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella* spp by PCR. *Appl Environ Microbiol.* 3735-3737.
37. Saleh IA (2005) Studies on milk production of buffaloes. Ph. D. thesis, Fac. of Agric. Mansoura Univ. Egypt
38. Schalm OW, Noorlander DO (1957) Experiments and observation leading to development of the California mastitis test. *J. Am. Vet. Med. Assoc.*130:199–204.
39. Schalm OW, Carrol, Jain NC (1971) Bovine mastitis. 1<sup>st</sup> Ed. Lea and Febiger, Philadelphia, USA.
40. Selem RS, Rashed Amany YM, Fahmy BGA (2002) Mastitis pathogens: attachment-related virulence features, whey protein markers and antibiotic efficacy in cows. *Vet. Med. J. Giza* 50:405. 17.
41. Shoshani E, Leitner G, Hanochi B, Saran A, Shpigel N, Berman A (2000) Mammary infection with *Staphylococcus aureus* in cows: progress from inoculation to chronic infection and its detection. *J. Dairy Res.*67:155.
42. Taponen S, Salmikivi L, Simojoki H, Koskinen MT, Pyörälä S (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J. Dairy Sci.*92:2610–2617.
43. Toye B, Woods W, Bobrowska M, Ramotar K (1998) Inhibition of PCR in genital and urine specimens submitted for *Chlamydia trachomatis* testing. *J.Clin.Microbiol.*36:2356–2358.
44. Varshney JP, Naresh R (2004) Evaluation of a homeopathic complex in the clinical management of udder diseases of riverine buffaloes. *Homeopathy* 93: 17–20.

7/7/2010