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RAPID DIAGNOSIS OF SUBCLINICAL STAPHYLOCOCCUS AUREUS-MASTITIS IN DAIRY BUFFALOES USING PCR TECHNIQUE

(With 4 Tables and 2 Figures)

By

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التشخيص السريع لالتهاب الضرع التحت السريري في الجاموس الحلاب الناتج عن عدوي المكور العنقودي الذهبى بأستخدام تقنية سلسلة التفاعل المتبلمر

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أجريت الدراسة على عدد ثلاثون جاموس حلاب يتراوح أعمارها من ثلاث إلى خمس سنوات حيث تم إختيارة عشوائياً من مزرعة كلية الطب البيطري جامعة قناة السويس وذلك لفحصه معملياً ضد مرض التهاب الضرع التحت السريري الناتج عن عدوي جرثومة المكور العنقودي الذهبى بأستخدام أختبار الكاليفورنيا وطرق الزرع البكتريولوجي وكذلك تقنية سلسلة التفاعل المتبلمبر. وتبين من الفحص بأستخدام أختبار الكاليفورنيا أن عدد الأرباع المصابة بالمرض كان ٣٠ ربع حلاب وبنسبة ٢٩,٤١% ممثلاً لعدد ١٨ جاموسة بنسبة ٦٠%. وأكدت النتائج المتحصل عليها دقة وحساسية وفعالية تقنية سلسلة التفاعل المتبلمر في تشخيص المرض وذلك بالمقارنة بالطرق البكتريولوجية المعملية التقليدية وأثبتت الدراسة أن جرثومة المكور العنقودي الذهبى لها حساسية ضد المضادات الحيوية التالية: الأمبسلين والأموكساسيلين والكلوكساسلين والجنتاميسين والأموكساسيلين مع حمض الكلافيولنيك وكذلك الأوكسى تتراسيكلين والبنسلين. وبعد تطبيق برنامج السيطرة على المرض لمدة شهركانت نسبة الشفاء ٢٥% عن طريق التقييم بأستخدام طرق الزرع التقليدية ونسبة ٣٦,٣٦% بأستخدام تقنية سلسلة التفاعل المتبلمر وعلية فمن وجهة النظر الأقتصادية وللسيطرة على ذلك المرض يفضل التخلص من الجاموس الحلاب الذي لا يستجيب للعلاج وكذلك يفضل الحرص عند أستخدام المضادات الحيوية لعلاج المرض حتى يتم تجنب إفراز سلالات من المكور العنقودي الذهبى لها مناعة ضد العلاج ومشكلة تواجد بقايا المضادات الحيوية في الألبان وذلك لضمان صحة وسلامة المستهلكين.

SUMMARY

A total of 30 milking buffaloes, 3 to 5 years age, were randomly selected from farm of Faculty of Veterinary Medicine-Suez Canal University for

investigation of *Staphylococcus aureus* subclinical mastitis by California mastitis test (CMT), culture method and Polymerase Chain Reaction (PCR). The detected cases were isolated and a controlling attempt was carried out prior and post milking processes. The total number of quarters affected by subclinical mastitis using CMT was 30 (29.41%) representing 18 buffaloes (60%). PCR is rapid technique for detection of Staphylococcal subclinical mastitis when compared with culture methods. *S. aureus,* was sensitive to ampicillin, amoxicillin, cloxacillin, gentamicin, amoxicillin + clavulinic acid, oxytetracyclin and pencillin G. After application of control program for one month, the cure rate was 25% as evaluated using culture methods and 36.36% as evaluated using PCR assay. From the economic point of view and diseases control, it could be recommended that culling of dairy buffaloes suffering from *Staphylococcus aureus* subclinical mastitis that did not respond to treatment is a better choice, also the caution and restricted prescription is recommended to avoid development of resistant bacteria and to avoid antibiotic residues in milk.

Key words: *Mastitis, dairy buffaloes, Staph aureus, PCR.*

INTRODUCTION

Mastitis is an inflammation of the mammary gland usually caused by infectious pathogen which commonly enters the udder mainly through the teat orifice. *Staphylococcus aureus* appear to be the most common mastitis pathogens causing clinical and subclinical mastitis of dairy animals (Holdway, 1992). *Staphylococcus aureus* infection is characterized by intermittent shedding from the infected udder and consequently the bacteriologically negative results of the examined milk may do not guarantee that a cow is free of infection (Radostits *et al.*, 2000). *S. aureus* in milk usually cause serious food poisoning in many consumers according to Food and Drug Administration (FDA 1995). In spite of a large research efforts on epidemiology and control for subclinical mastitis, was carried out, the clinical occurrence of this disease remains a substantial problem for dairy producers (Booth, 1995). From the economic point of view, subclinical mastitis reduces the milk yield by a level ranging from 10-20 % depending upon the extent duration of infection in addition to changes which may occur in milk constituents that render it of low quality and unfit for processing (Holdway, 1992).

Dry therapy and post-milking teat disinfectants reduce the prevalence of contagious pathogens, like *S. aureus*. However, complete bacteriological cure of the affected udder is rarely achieved and great attention has been given for diagnosis of subclinical mastitis by rapid screening tests as California Mastitis Test (CMT) (Schalm *et al.,* 1971). California Mastitis Test is recommended as screening test for detection of subclinical mastitis on dairy farms (Viani *et al.,* 1990). The diagnosis of staphylococcal infections is a time-consuming process that is generally dependent on the phenotypic characterization of cultured bacteria. However, an increasing number of investigators have employed the tools of molecular biology to facilitate the diagnostic process. Based on its speed and sensitivity, the preferred approach has been the use of PCR to amplify specific target genes (Greisen *et al.,* 1994). Many tests have been developed for the diagnosis of subclinical mastitis such as screening indirect tests and bacterial cultures. Polymerase chain reaction (PCR) assay is considered less time-consuming, rapid, specific identification method, and can discriminate between closely related organisms, such as *S. aureus* and *Streptococcus uberis* (Berri *et al.,* 2000).

The objective of this study was carried out to use the Polymerase chain reaction as a rapid method for diagnosis of *Staphylococcus aureus* subclinical mastitis in dairy buffaloes in comparison with culturing procedure. Field trial for control of *S. aureus* in a dairy farm was also carried out.

MATERIALS AND METHODS

Animals

A total of 30 dairy buffaloes, 3 to 5 years age, were randomly selected from Suez Canal University farm, Faculty of Veterinary Medicine at Ismailia governorate. The udder and teats of the selected buffaloes were physically and clinically investigated to exclude the clinical mastitis. All animals in the farm were hand milked, twice daily.

Milk samples

Udders and teats of the selected buffaloes were thoroughly washed and dried with sterile clean towel and disinfected with 70% alcohol. The first milking streams were rejected and 50 ml of milk / each quarter was collected in sterile screw capped bottle. The collected sample was divided into two portions; one portion was subjected to CMT. The other one was subjected to bacteriological examinations and PCR analysis.

Screening test

A total of 102 milk samples were subjected to California mastitis test (CMT) for detection of subclinical mastitis. The positive samples were subjected to bacteriological examinations and PCR analysis.

Bacteriological examination

The milk samples were incubated for 18-24 hours at 37°C, and then 10 ml of milk samples were transferred into sterile small centrifuge tubes. The tubes were centrifuged at 3000 rpm for 20 minutes, and then the cream and the supernatant were discarded to obtain sediment. The sediment was streaked on the surface of Nutrient agar, Mannitol salt agar and blood agar. The inoculated plates were incubated for 24-48 hours at 37°C. The suspected colonies were purified and primarily identified by Gram stain, catalase and coagulase tests. The positive isolates were propagated on Baired Parker agar for further examination. The biochemical identification of the isolated strains was carried out according to Collee *et al.* (1996) and Quinn *et al.* (2002).

Antibiotic sensitivity testing

The bacterial isolates were tested for their resistance using disc diffusion method according to Bauer *et al.* (1966), while, Zones of inhibition were determined according to National Committee for Clinical Laboratory Standards (NCCLS, 2002). Mannitol salt agar and Nutrient agar were the media used and the antibiotic discs used were ampicillin (10µg/disc), amoxicillin (25µg/disc), cloxacillin (5µg /disc), gentamicin (10µg /disc), amoxicillin + clavulinic acid (3µg /disc), penicillin G (10 IU/disc) and oxytetracycline (30µg /disc), that were supplied from Oxoid (Cambridge, CB5 8BZ, UK) and commonly used in treatment of clinical and subclinical mastitis. The diameter of inhibition zones were measured for each plate and the isolates were categorized as susceptible and resistant.

Polymerase chain reaction (PCR) assay

The extraction of DNA from the milk samples was carried out according to Riffon *et al.* (2001) and Meiri-Bendek *et al.* (2002), while the DNA extraction from the bacterial isolates was done according to Sritharan and Barker (1991). The PCR reactions were carried out in a final volume of 50µl in PCR tubes, the reaction mixture consists of 1 µl (200 ng) of the extracted DNA template from the bacterial isolates or 5 µl of the extracted DNA from the milk samples, 5 µl of 10X PCR buffer (75M Tris-HCL, pH 9.0, 2mM $MgCL_2$, 50mM KCL, 20mM $(NH_4)_2SO_4$), 2 µl dNTPs (10mM) (Biotools), 1µl Ampli Taq DNA polymerase (Bio tools) and 0.5 µl (50 pM) from the forward primer

(GTA GGT GGA AAG CGT TAT CC) and reverse primer (CGC ACA TCA GCG TCA G) (Integrated DNA Technologies, Inc. Coralaville) of the 16S rRNA gene of *S. aureus* according to (Greisen *et al.*, 1994). The volume of the reaction mixture was completed to 50 µl of DDW. The thermal cycler (Omni-Gene, USA) was adjusted as follows; initial denaturation 94°C for 4 minutes, followed by 35 cycles of 1minute denaturation at 94°C, 1 minute annealing at 52°C and 1 minute extension at 72°C, followed by final 72°C extension for 10min, then hold at 4°C. Five micro liters of PCR product was electrophoresed on a 1% agarose gel stained with 0.5ug of ethidium bromide/ml to determine the size of the product. Negative control, positive control (kindly supplied from Microbiology Department, Fac. Vet Med., Cairo University) and 100bp molecular DNA marker (Promega, Madison, WI, USA) were included in each PCR run at a constant current of 40 V for 1 hour. The negative control consisted of all PCR components except the template DNA. If negative control became positive, the entire PCR was repeated. The gels were visualized under U V illumination (Eagle Eye II, Startagene, Germany) and thereafter photographed using digital camera. The sizes of the amplified product were determined by comparison to DNA marker.

Control

The examined buffaloes were grouped as subclinically free and subclinically positive cases. The subclinically free cases were milked before the positive cases prior hand milking of all animal groups, each teat was washed with a sanitizing solution (alcohol 70%) and dried by a disposable paper. Post-milking, teat dip technique was used by iodophore 4% (Radostits *et al.*, 2000). All equipments and utensils used for milking process were thoroughly washed and disinfected post-milking. For subclinically positive cases, each syringe (5g) containing 75mg ampicillin as sodium salt and 200mg cloxacillin as sodium salt (®Lactaclox intra mammary suspension-Norbrook Laboratories Limited Newry BT35 6JP) was used against *S. aureus*, two infusion tubes were administered twice daily with 12 hours apart for five days in the affected quarter, and the milk of all quarters were discarded. All new introducing buffaloes to the milking herd were cultured for *S. aureus* and the detected cases were received the recommended antibiotic therapy. One month later, milk samples were subjected to bacteriological examinations and PCR analysis for detection of subclinical *S. aureus* mastitis.

RESULTS

Table 1: Results of California Mastitis test (CMT) of examined milk samples collected from dairy buffaloes (n=30).

	Total No.	Positive samples				%	Negative samples	%
		+++	++	+	trace			
Samples No.	102	5	8	13	4	29.41	72	70.59
Buffaloes No.	30	18				60	12	40

Table 2: Positive and negative examined cases for subclinical *Staphylococcus aureus* mastitis examined by different methods.

Method	Total	Positive		Negative	
		No.	%	No.	%
CMT	30	18	60	12	40
Culture Method	18	8	44.44	10	55.56
PCR	18	11	61.11	7	38.89

Table 3: Inhibition zone diameter (IZD) of test antibiotics against different isolates of *S. aureus* isolated from milk samples.

Strain code	Co	TE	AP	PN	GN	AX	AC
S-1	S^{++}	R	R	R	R	R	S^{++}
S-2	S^{++}	S^{++}	S^{++}	S^{++}	S^{++}	S^{++}	S^{++}
S-3	S^{+-}	S^{+-}	R	R	R	S^{++}	S^{+--}
S-4	R	S^{++}	S^{-}	R	S^{+}	S^{--}	S^{---}
S-5	S^{++}	S^{+++}	S^{++}	R	S^{-}	S^{++}	S^{+++}
S-6	S^{++}	S^{+}	S^{+}	R	R	S^{-+}	S^{++}
S-7	S^{++}	S^{-}	S^{++}	S^{+}	S^{++}	S^{-+}	S^{+}

* CO, cloxacillin; TE, tetracycline; AP, Ampicillin; PN, penicillin G; GN, gentamycin; AX, Amoxil and amoxilclave. AC

*0 to 5 mm Resistance (R) 5 to 15 mm Sensitive (S^{+}) 15 to 25 mm moderately Sensitive (S^{++}) more than 25 mm highly Sensitive(S^{+++})

Table 4: Evaluation of control results based on bacteriological examination and PCR for collected milk samples from treated buffaloes.

Method	Total No. of buffaloes	Control				Cure rate	
		Before		After			
		No.	%	No.	%	No.	%
PCR	11	11	100	7	63.64	4	36.36
Culture	8	8	100	6	75	2	25

Fig. 1: Results of subclinical *Staphylococcal aureus* mastitis examined by CMT, culture method and PCR.

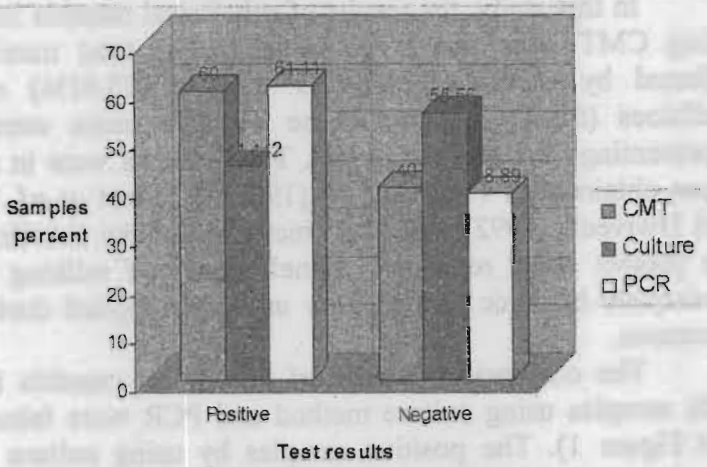
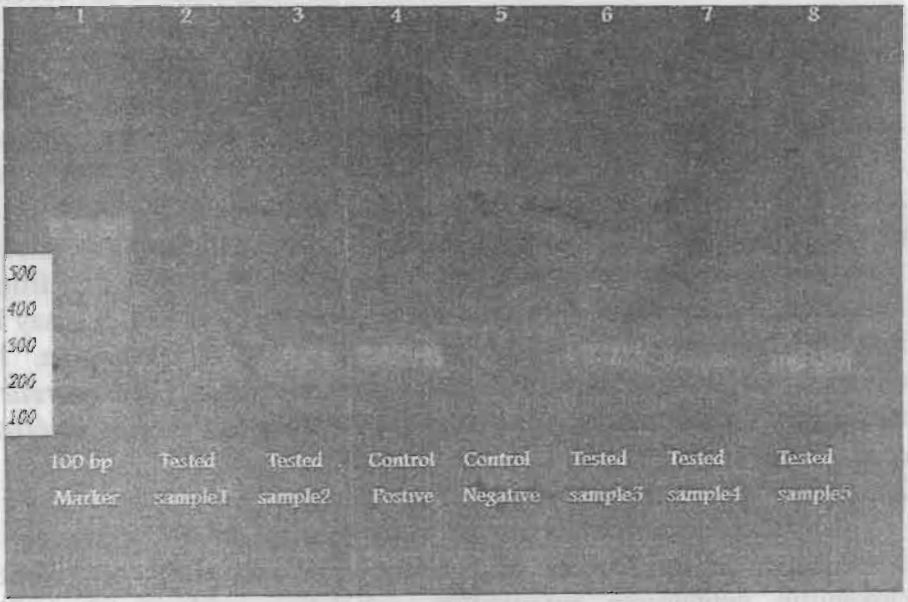


Fig. 2: Gel electrophoresis 1% image of amplicon produced from 16 Sr RNA (228 bp) PCR assay.



DISCUSSION

1. Subclinical Mastitis Detection

In this study, the results of subclinical mastitis in dairy buffaloes using CMT were shown in Table 1. The total number of quarters affected by subclinical mastitis was 30 (29.41%) representing 18 buffaloes (60%), meanwhile the negative cases were 72 (70.59%) representing 12 buffaloes (40%). These results were in agreement with those obtained by Patgiri *et al.* (1978); Didonet *et al.* (1988); Behera and Dwivedi, (1992). The high incidence of the subclinical mastitis in the present study may due to inefficiency of milking personnel, and inadequate hygienic and sanitary measures applied during the milking processes.

The comparison results of subclinical mastitis for 18 positive milk samples using culture method and PCR were tabulated (Table. 2 and Figure 1). The positive samples by using culture method for *S. aureus* was 8 (44.44 %) out of 18 samples These results were coincided with El-Balkemy *et al.* (1997) and El-Attar *et al.* (2002) who reported that the percentage of positive bacteriological samples was 57.9% and the isolated microorganisms were mostly *S. aureus, strept agalactiae, strept dysgalactiae* and *Escherichia coli.* The results of PCR revealed that the positive samples for *S. aureus* were 11 (61.11 %) out of 18 samples (Figure, 2). The current study confirmed that PCR assay is accurate method for detection of *S. aureus* subclinical mastitis when compared with culture methods. These results were coincided with results obtained by Berri *et al.* (2000) and Yamagishi *et al.* (2007) who stated that the PCR assay could detect concentrations of *S aureus* as low as one colony-forming unit/ml milk. Among 106 milk samples collected from individual quarters of lactating cows in one dairy herd and from a bulk tank, *S aureus* was detected in nine samples by the PCR assay but in only three samples by conventional microbiological culture. The obtained results might be explained due to the behaviour of *S. aureus* as it lives intracellular and their intermittent shedding in the milk (Radostits *et al.,* 2000).

2. Antibiotic sensitivity testing

Antibiotic sensitivity test indicated that the isolated strains (n= 7) were have varying degree of sensitivity to ampicillin, amoxicillin, cloxacillin, gentamicin, amoxicillin + clavulinic acid, oxytetracycline and penicillin G as shown in Table 3. Two isolates out of seven showed moderate degree of sensitivity against penicillin G and these results were

in agreement with those of Taha, (2002) and Kalsoom *et al.* (2004). Most cephalosporins and cloxacillin are effective against B-lactamase-producing *Staphylococci* Fthenakis, (1998) but ampicillin + cloxacillin have the additional advantage of being effective against *Streptococci* and *Escherichia coli* (Norbrook Laboratories Ltd Station Words, Norwey) and thus are useful in the early treatment of acute mastitis, where the causative agent has not been identified.

3- Control of Subclinical Mastitis

The results of control program for *S. aureus* subclinical mastitis are shown in Table 4. The cure rate was 25% as evaluated using culture methods and 36.36% as evaluated using PCR assay, which might be attributed to intracellular nature of the pathogen, late infection, previous abusing of antibiotic therapy and numbers of affected quarters. This result was in agreement with Sol *et al.* (1997) who reported that cures were only 34% when 89 cows in 10 Dutch herds were treated for *S. aureus* subclinical mastitis and disagreed with Gonzalo *et al.* (2004) who stated that cure rates ranged between 65.0 and 95.8%, Nevertheless, there is still a considerable percentage of animals re-infected, with consequences in the quantity and quality of milk production (Gonzalo *et al.,* 2002). In addition, Roberson *et al.* (1994) reported that mastitis caused by *S. aureus* was extremely difficult to control by treatment alone. Successful controlling programme is only gained through prevention of new infections, cow culling, efficient hygienic and sanitary measures during milking processes. Another opinion was stated by Wilson *et al.* (1995) who reported that segregation of staphylococcal infected animals has been proven to significantly reduce the prevalence of staphylococcal mastitis and bulk tank somatic cell counts.

There are several factors influence the cost of such a plan of control including treatment and milk loss due to the withdrawal period after antibiotic treatment (milk for human consumption may only be taken from 3 days after the last treatment and cattle may be slaughtered for human consumption only after 4 days from the last treatment). On the other hand, there is the additional milk produced due to subclinical mastitis treatment. Additionally, the long-term benefits in the next lactating period from the improvement of udder health status in animals that will enter the dry period may play another role.

In conclusion *S. aureus* subclinical mastitis was common in dairy buffaloes at Faculty Vet. Med. farm, Ismailia, that affecting milk yield (quality and quantity) and consumer safety. CMT in combination with culture method was not enough for detection of *S. aureus* subclinical

mastitis. PCR assay is accurate, rapid and specific method for detection of *S. aureus* subclinical mastitis in dairy buffaloes especially in early stages whereas the treatment will be more effective. Economically, it could recommend that culling of dairy buffaloes with *S. aureus* subclinical mastitis and did not response to treatment, appear to be is a better choice. The caution and restricted prescription is recommended in order to avoid development of resistant bacterial strains and to avoid antibiotic residues in milk.

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