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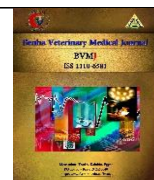

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Bacteriological and molecular diagnosis of most common bacteria causing subclinical mastitis in cow

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ABSTRACT

The aim of the study was using polymerase chain reaction (PCR) technique for detecting the most bacteria causing mastitis in cows as streptococci spp., *Escherichia coli* and *Staphylococcus aureus* by using Genus specific primers for each one. One hundred milk samples were collected from cases of subclinical bovine mastitis positive for California Mastitis Test (CMT). The samples were bacteriologically cultivated on to 5% sheep blood agar and also, on different types of media (MacConkey's agar plates, Edward's agar medium and mannitol salt agar) to isolate the major bacteria causing mastitis and identified biochemically. Finally, these bacteria were identified by using PCR technique with a specific primer for each genus. The PCR results revealed that *E. coli* 16/46 (34.7%), *Staphylococcus aureus* (*S. aureus*) 12/40 (30%) and streptococci spp. 11/39 (28.2%). Multiplex PCR for typing of streptococci spp. revealed that *St. agalactiae* 3/39 (7.69%) and *St. dysgalactiae* 8/39 (20.51%). In conclusion, in the present study, PCR analysis was used for diagnosis of major bacteria causing subclinical mastitis in cows by using specific primers for each one. The most prevalent bacteria causing subclinical mastitis were *E. coli, S. aureus, S. dysgalactia and S. agalactia*.

1. INTRODUCTION

Bovine mastitis is the most important disease in dairy cattle that causes high economic losses due to drop in milk yield and increased treatment costs (Koskinen et al., 2009). Many bacterial pathogens are responsible for mastitis in dairy animals such as *Escherichia coli*, Corynebacteria spp., Staphylococci spp., Pseudomonas spp., streptococci spp., Mycoplasma, and Klebsiella spp. (Radostits et al., 1995; Haas et al., 2005; Yuan et al., 2011).

S. aureus is considered as a main bacterium which causes subclinical mastitis in dairy cows leading to high economic losses worldwide (Godden et al., 2002).

Streptococcus agalactiae, S. uberis, and *S. dysgalactiae* are prominent Gram-positive, catalase-negative cocci (PNC) pathogens that cause bovine mastitis. In the case of *S. uberis,* a high prevalence has been reported throughout the world (Phuektes et al., 2001; Bradley et al., 2007). Intra mammary infection (IMI) caused by *S. agalactiae* has been rarely observed during the last decades in Switzerland, likely because it can be controlled by improved milking management and it shows good susceptibility to antibiotics (Guelat-Brechbuehl et al., 2010). A recent publication, however, suggests that *S. agalactiae* may regain clinical importance, particularly in herds milked by automatic milking systems (Mweu et al., 2012).

The most prevalent bacteria isolated from subclinical mastitis cases were. *Staphylococcus* species (57.14%) dominated followed by *Streptococcus* species (28.57%) and *E. coli* (14.29%) (Mekonnin et al., 2016).

On bacteriological examination, examined 127 CMT positive cows' samples, 49 (38.58%) samples were positive for streptococcus species with 21 (16.5%) *Strept. agalactiae*, 15 (11.8%) *Strept. uberis* and 13 (10.2%) *Strept. dysgalactiae* were identified (Amin et al., 2017). Therefore, PCR depends on using 16S rRNA or 23S rRNA regions to consider a most accurate, reliable and fast method for the identification of different bacteria (Bes et al., 2000; Sabat *et al.*, 2000).

The aim of the current work is isolation of the major bacteria causing mastitis and detected by PCR technique using 16SrRNA specific primer for each genus.

2. MATERIAL AND METHODS

2.1. Sampling:

A total number of 100 subclinical mastitis milk samples (positive for CMT) (APHA 2001) were collected aseptically for bacteriological examination according to the procedures of Radostits et al. (2007).

A 15-20 ml of milk sample was put in a clean sterile screw capped bottle and labeled. The milk samples were kept in sterile container till transported to the laboratory. The samples were incubated at 37 °C for 24 hrs in an incubator then submitted for the bacteriological examination.

2.2. Isolation:

The milk samples pre-incubated at 37 °C for 18-24 hrs, then a loopful from incubated milk was streaked onto 5% sheep blood agar, MacConkey's agar plates, Edward's agar medium and mannitol salt agar, then incubated aerobically

at 37 °C for 24-48 hrs. The suspected colonies were picked up, sub-cultured onto nutrient agar slants then incubated aerobically at 37 °C for 24 hrs (Quinn et al., 2002).

2.3. Identification of the bacterial Isolates:
The suspected colonies were examined for their gross appearance (morphological characteristics) including the colony shape, size, color of the colonies or the pigment production, surface texture (rough or smooth), the consistency (mucoid or non-mucoid), type of α, ß or γ type of hemolysis) onto the blood agar, and the metabolic activity onto MacConkey's agar (lactose fermenter (LF) or non-lactose fermenter (NLF). The suspected colonies were examined microscopically using Gram stained films before transferred onto slope agar for further identification using biochemical tests according to Quinn et al (2002).

2.4. Molecular analysis of bacterial isolates:
2.4.1. DNA extraction from S. aureus and Streptococci spp.
DNA extraction from them was carried out by using multi-genomic DNA extraction kit (Fermentas, Germany) according to manufacturer's instructions for each one of them.

2.4.2. DNA extraction from E. coli isolates:
DNA extraction from *E. coli* isolates was carried out by using boiling method as following; an overnight bacterial culture (200 µl) was mixed with 800 µl of distilled water and boiled for 10 min. The resulting solution was centrifuged at 10000 rpm for 10 min and the supernatant used as the DNA template (Ahmed et al., 2007).

2.4.3. Polymerase Chain Reaction of all isolates:
The PCR of *S. aureus*, streptococcus spp. and *E. coli* was carried out in a 25-µl reaction mixture containing; 5 µl of DNA of isolates, 12.5 µl of 2 Master Mix1 (Intron), 1.25 µl each of the forward and reverse primers (20 pmol/µl) (Sigma, USA) specific for each bacteria, (Table 1) and distilled water was added to complete the final volume to 25 µl. All bacterial isolates were submitted to PCR amplification according to condition of each one as described in table (2).
The multiplex PCR of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* was carried out in a 25-µl reaction mixture containing; 5 µl of DNA of isolates, 12.5 µl of 2 Master Mix1 (Intron), 0.75 µl of the forward and reverse primers of each one (20 pmol/µl) (Sigma, USA) specific for each bacteria, (Table, 1) and distilled water was added to complete the final volume to 25 µl. All bacterial isolates were submitted to PCR amplification according to condition of each one as described in table (2).
The PCR products were analyzed through gel electrophoresis on a 1.5% or 2% agarose gel prepared with Tris/Acetate/EDTA (TAE) buffer plus agarose powder stained with 0.5 µg/ml ethidium bromide (Sambrook et al., 1989). A 10 µl aliquot of a molecular marker containing a DNA Ladder (Intron) was added to the first well of each gel and 10 µl of samples in the other wells at 80 V for 1 hr. After one-hour electrophoretic run, the single DNA bands were visualized by ultraviolet transillumination according to target size of each gene as showed in table (2).

3. RESULTS

3.1. Incidence of bacterial isolates in the samples:
One hundred subclinical mastitis milk samples (positive for CMT) were examined bacteriologically and identified biochemically showed *E. coli* 46/125 (36.8%), *S. aureus* 40/125 (32%), *Streptococcus* 39/125 (31.2%) as shown in table (3).

Table 1 Oligonucleotide primers used for amplification of specific genes:

Target	Primer Name	Oligonucleotide Sequence (5' - 3')	Amplicon size (bp)	Reference
S. aureus	S. aureus-F	AACCTTTGTTGGTAGACATCCTCCACG	108 bp	Martinovu *et al*. (1996)
	S. aureus-R	CGTAATGACATTTCAGTAGATAATACAACA		
E.coli	ECO-f	GACCTCGGTTTAGTTCACAGA	585 bp	Alireza *et al*. (2010)
	ECO-R	CACACGCTGACGCTGA		
Streptococci species	C-1	GCGTGCCTAATACATGCAA	207 bp	Maisak *et al.* (2007)
	C-2	TACAACGCAGGTCCATCT		
Streptococci agalactiae sklA3 (fibrinogen binding protein)	GSag-S	ATT GAT AAC GAC GGT GTT ACT GT	487 bp	Andreas *et al.* (2013)
	GSag-AS	CAT AGT AGC GTT CTG TAA TGA TGT C		
Streptococcus dysgalactiae 16S rRNA	GSdys-S	GTG CAA CTG CAT CAC TAT GAG	279 bp	
	GSdys-AS	CGT CAC ATG GTG GAT TTT C		
Streptococcus uberis pauA (plasminogen activator A)	GSub-S	TGA TTC CGA CTA CTA CGC TAG AT	723 bp	
	GSub-AS	ATA CTT TGA GTT TCA CCG AGT TC		

Table 2 General PCR conditions of bacterial isolates:

	Gene	Hot start	Denaturation	Anneal.	Prim. ext.	Cy.	Final ext.	Target
1	*Staph aureus*							
	SU-F SU-R	92 °C/2 min	95 °C/30 sec	55 °C/30 sec	72 °C/30 sec	30	72 °C/10 min	108 bp
2	*Streptococcus spp.*							
	C-1 C-2	94 °C/4 min	94 °C/1 min	55 °C/1 min	72 °C/1 min	30	72 °C/10 min	207 bp
3	*E. coli*							
	ECO-F ECO-R	95 °C/3 min	94 °C/45 sec	58 °C/45 sec	72 °C/1 min	30	72 °C/3 min	585 bp
4	Multiplex PCR of *St. agalactia, St. dysgalactia and St. uberis*							
	GSag-S GSag-AS	95 °C/15 min	94 °C/1 min	58 °C/1 min	72 °C/1 min	35	72 °C/10 min	487 bp
	GSdys-S GSdys-AS	95 °C/15 min	94 °C/1 min	58 °C/1 min	72 °C/1 min	35	72 °C/10 min	279 bp
	GSub-S GSub-AS	95 °C/15 min	94 °C/1 min	58 °C/1 min	72 °C/1 min	35	72 °C/10 min	723 bp

Table 3 Bacterial species on the examined sample

Bacterial isolates	Number	Incidence
S. aureus	40	32 %
E. coli	46	36.8%
Streptococcus	39	31.2 %
Total	125	

Percent (%) is according to bacteriologically total isolates.

3.2. Molecular detection of the different biochemically identified bacterial isolates:

The molecular detection of isolates by using PCR with specific primers for each genus (Table 4) confirmed that 12/40 strains of *S. aureus* were positive with a percent of (30%) at molecular size 108 bp, as shown in Fig. (1). Regarding to *E. coli* there were 16/46 strains positive at rate of (34.7%) at molecular size 585 bp as shown in Fig. (2). For Streptococci spp. 11/39 with a percent of (28.2%) at molecular size 207 bp as shown in Fig. (3).

Table 4 Molecular detection of the different biochemically identified bacterial isolates (By PCR):

Recovered bacteria species	Total number of bacterial isolates (Biochemically)	PCR result	
		Number	Percentage %
S. aureus	40	12	30 %
E. coli	46	16	34.7 %
Streptococcus spp.:	39	11	28.2 %
S. agalactia		3	7.69 %
S. dysgalactia		8	20.51 %

Percent (%) according to biochemically total number of bacterial isolates for each genus.

3.3. Multiplex PCR for identification of *Streptococcus* species

Streptococci isolates obtained from examined samples biochemically (39 isolates), which were examined by multiplex PCR to identify the species.

Multiplex PCR protocol revealed that only 8/39 isolates (20.51%) of *S. dysgalactiae* and 3/39 isolates (7.69%) of *S. agalactiae* were detected from subclinical mastitis milk samples. No *S. uberis* isolates were detected from subclinical mastitis milk samples.

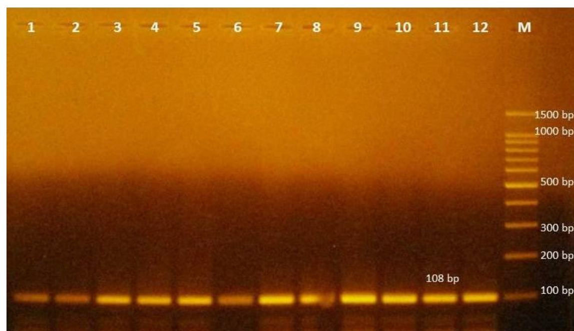

Figure 1 Amplification products of *S. aureus* strains analyzed by electrophoresis on 2% agarose gel. Lane M: 100-bp DNA ladder, Lane (1-12): positive *S. aureus* at size 108 bp

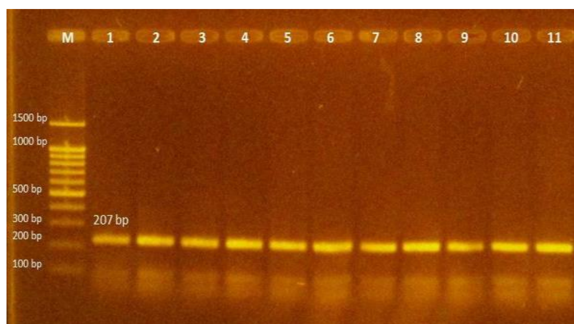

Figure 2 Amplification products of *Streptococci* spp. strains analyzed by electrophoresis on 2% agarose gel. Lane M: 100-bp DNA ladder, Lane (1-11): positive Streptococcus spp. at size 207 bp.

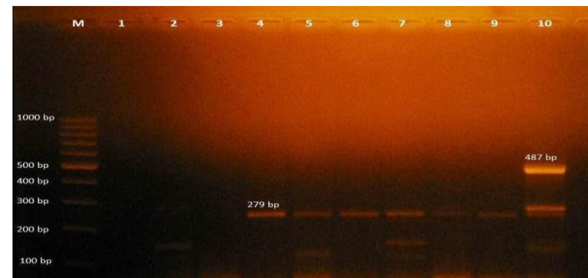

Figure 3 Amplification products of multiplex PCR of Streptococci spp. analyzed by electrophoresis on 1.5% agarose gel. Lane M: 100-bp DNA ladder, Lane (1, 2, 3, 8, 9): negative, lane (4, 5, 6, 7): positive *Streptococcus dysgalactia* at size 279 bp and lane (10): positive *Streptococcus agalactia* at size 487 bp and *Streptococcus dysgalactia* at size 279 bp.

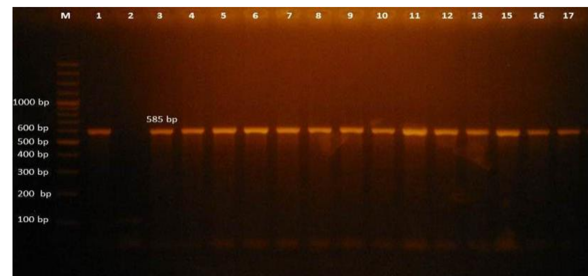

Figure 4 Amplification products of *E. coli* spp. analyzed by electrophoresis on 1.5% agarose gel. Lane M: 100-bp (plus) DNA ladder , Lane (1) and (3-17): positive *E. coli* at size 585 bp and lane (2): negative.

4. DISCUSSION

PCR is highly sensitive to a few picogram amounts of nucleic acid is enough to identify the organism, allowing the elimination of culture (Whitehead and Cotta, 2000). In our study, we examined a total number of 100 milk samples of subclinical mastitis (positive with California mastitis test) bacteriologically and identified biochemically, the isolated bacteria were *S. aureus*, *E. coli* and streptococci spp.

Among subclinical mastitis milk samples, *E. coli* was the major pathogen with a percentage of (36.8%) followed by *S. aureus* (32%) and Streptococci spp. (28.2%). The previous results were similar to Calderon and Virginia (2008) and Hande et al. (2015), while Pradhan et al. (2011) reported that the major pathogen of subclinical mastitis was *S. aureus* (34.01%) followed by *E. coli* in (33.33%) and Streptococci spp. in (8.163%). A higher incidence of *S. aureus* (32%) was isolated from subclinical mastitis as (52.2%) by Abdel-Rady and Sayed (2009), (35.71%) by Abera et al. (2013) and (26.08%) by Hande et al. (2015).

Streptococci spp. as *S. agalactie*, *S. dysgalactiae,* and *S. uberis* have been reported as a common causative agents for mastitis (Khan et al., 2003). PCR amplification of species-specific parts of the gene encoding the *16S rRNA* and *cfb* gene, had been successfully used for the rapid and reliable identification of these species (Jayarao et al., 1991; Picard *et al.*, 2004).

In the current study, the multiplex PCR used for typing of streptococci isolates showed *S. agalactiae* (7.69%) from subclinical mastitis, *S. dysgalactiae* (20.51%). The previous results are higher than Heba (2011), who isolated *S. agalactiae* with percent (3.1%) and S. *dysgalactiae* with (1.2%) from cases of subclinical mastitis.

Also, lower incidences of *S. agalactiae* were recovered by El-Zubeir et al. (2006), at rates of 0.83%. Lower isolation rate of *S. dysgalactiae* with 2.5% and 4% was recorded by Balakrishnan et al. (2004). On the other hand, higher incidences of *S. agalactiae* isolated from mastitis of cows were recovered by Borkowoska et al. (2006) and Bi et al. (2016) with isolation rates of 84.8% and 92.2%, respectively.

5. CONCULOSIONS

In the present study, PCR analysis was used for diagnosis of major bacteria causing subclinical mastitis in cows by using specific primers for each one. The most prevalent bacteria causing subclinical mastitis were *E. coli, S. aureus, S. dysgalactia and S. agalactia*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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