



Differentiation between *streptococcus agalactia*, *streptococcus dysagalactia* and *staphylococcus aureus* isolated from milk of mastitic cows by polymerase chain reaction.

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ABSTRACT

This study was conducted for bacteriological examination of 130 milk samples collected from clinically mastitic cows in different areas at Menoufyia governorate . The results showed that *streptococcus agalactia* ,*streptococcus dysagalactia* and *staphylococcus aureus* were isolated with percentage (11.5, 2.3% an 10%) respectively. Polymerase Chain Reaction (PCR) using specific primer were used as an accurate and cost- effective molecular diagnostic technique of identifying mastitis pathogens.

Keywords: *streptococcus agalactia*, *streptococcus dysagalactia*, *staphylococcus aureus*

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1. Introduction

Bovine Mastitis (BM) is an inflammation of the mammary gland, usually due to a microbial infection (Watt 1988). Mastitis in dairy cows is among the most important diseases worldwide and causes considerable economic loss to dairy industry. These losses due to lower milk yields , reduction milk quality and higher production costs (Awale et al., 2012 and Joshi and Gokhle 2006) Bacteria that commonly cause mastitis are generally classified as either 'contagious' or 'environmental' pathogens, depending on the source of the pathogen and mode of transmission. The main contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*. These pathogens have adapted to survive within the mammary gland and are spread from cow to cow at or around the time of milking (Bradley 2002). *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma* species are considered as typical contagious pathogens .Typical environmental pathogens are called environmental streptococci (Gruet et al., 2001; Bradley

2002 and Barkema et al., 2009). Molecular diagnosis applied to mastitis problems on dairy farms because it is accurate and cost effective methods of identifying mastitic pathogens, surveillance and control of this economically important disease among dairy cows (Reyher and Dohoo 2011; Jones et al ., 2003 and Zhao et al., 2006). Therefore, the aim of the present work was to isolation and identification of *streptococcus agalactia*, *streptococcus dysagalactia* and *staphylococcus aureus* from mastitis cow's milk, and Identification of the isolates.

2. MATERIALS AND METHODS

2.1. Milk samples

130 milk samples are collected from clinically mastitic cows from different areas at El Menoufyia governorate under complete aseptic conditions. The udder ,teats and hands were washed with clean soap and water then washed with %70 ethyl alcohol .The first few steams of milk were rejected and about 20 ml of milk were

collected separately from each of the four quarters of the udder into sterile screw capped MacCartenys bottles. All samples were subjected to bacteriological examination. Bacterial culture: All milk samples were cultured on to blood agar and mannitol salt agar and edward agar media after 24 h incubation. It was incubated aerobically for 24-48 h .The bacterial

isolates were purified and identified according to (Baily and scott (1990) and koneman et al., (1992). Application of PCR for identification of *mecA* gene of *Streptococcus species* and *mecA* gene of *Staphylococcus species* was performed essentially by using Primers (Pharmacia Biotech) as shown in the following table 1.

Table (1): The sequence of Oligonucleotide primers specific for *streptococcus agalactia* , *streptococcus dysagalactia* and *staphylococcus aureus*:

Target gene	Oligonucleotide sequence (5' → 3')	Amplified products	Reference
<i>Strept. Sps.</i> tuf (F)	5' CAA CTT GAC GA AGGT CCT GCA '3	110	Prabhu et al. (2013)
<i>Strept. Sps.</i> tuf (R)	5' TGG GTT GAT TG AACC TGG TTT A '3		
<i>S. aureus</i> MecA (F)	5' TAG AAA TGA CTG AAC GTC CG '3	154	Martin et al. (2004)
<i>S. aureus</i> MecA (R)	5' TTG CGA TCA ATG TTA CCG TAG '3		

2.2. DNA extraction from bacterial culture (Cremonesi et al., 2006):

After overnight culture on blood agar plates, one or two colonies of purified bacterial isolates were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100°C for 20 minutes. From this suspension, a 5 ul aliquot was directly used as a template for PCR amplification.

2.3. DNA amplification reaction of *Streptococci* (Meiri-Bendek et al., 2002):

The PCR reaction mixture contained 2.5 µl of 10x Tag polymerase buffer (1.5 mM MgC12); 1 UL of each primer (10 pmol conc.); 1.0 µl of reverse primer (10 pmol); 0.2 µl of dNTP (25 mM), 0.1 µl of Taq polymerase (0.25U); 5 µl of DNA(50-100 ng/ µ l); add H2O (sterile) to total volume 25 µl. The reaction was carried out in a PCR thermal cycler as follows: denaturation for 4 min at 94°C, followed by 5 cycles of 94°C for 1 min., 72°C for 45 sec. each step; 20 cycles

of 94°C, 72°C for 45 sec. each step; and a step of 72°C for 5 min, at the end of the reaction. PCR products were fractionated on 1.5 % agarose gel stained with ethidium bromide.

2.4. DNA amplification reaction of *Staphylococci* (Jukes et al., 2010):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl of PCR mixture containing 3 µl of boiled cell lysate, 250 uM of each desoxynucleotide triphosphate, 1.5 U of Taq DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KC1 and 3 mM MgC12, Biotools), 1 uM of the primers *mecA*-R, *mecA*-F; 0.8 uM of *icaA*-R, *icaA*-F and 0.8 uM of *icaD*-R, *icaD*-F. Amplification conditions were: denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 1min, 58°C for 1 min and 72°C for 1 min, with final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% of agarose gel electrophoresis stained with ethidium

bromide and visualized and captured on UV transilluminator.

3. RESULTS

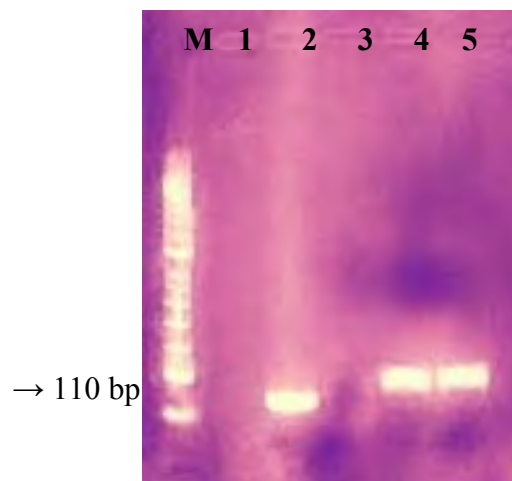
This study table (2) was conducted for bacteriological examination of 130 milk samples collected from clinically mastitic cows in different areas at El Menoufyia

governorate .The results showed that *streptococcus agalactia* ,*streptococcus dysagalactia* and *staphylococcus aureus* were isolated with percentage (11.5, 2.3% and %10) respectively . Table (1) indicate Polymerase Chain Reaction (PCR) by using specific primer used as an accurate and cost- effective molecular diagnostic technique of identifying mastitis pathogens

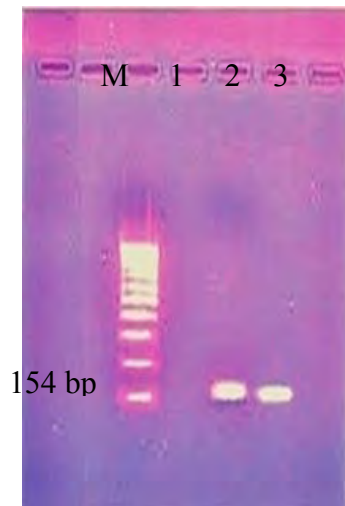
Table(2) Bacterial species isolated from milk samples of cows with clinical mastitis. (N. 130)

Microorganism	No.	% *
<i>Streptococcus agalactiae</i>	15	11.5
<i>streptococcus dysagalactia</i>	3	2.3
<i>Staphylococcus aureus</i>	13	10

* Percentage were calculated according to the number of samples examined.



Photograph (1): Agarose gel electrophoresis of PCR amplified products using specific *tuf* gene (*tuf* primers) of *Streptococcus* species. Lane M: 100 bp ladder as molecular DNA marker. Lane 1: Control negative for *tuf* gene of *Streptococcus* species. Lane 2: Control positive for *tuf* gene of *Streptococcus* species . Lane 3 {*Streptococcus ubris*): Negative finding for specific *tuf* gene . Lane 4 {*Streptococcus agalactiae*): Positive for specific *tuf* gene. Lane 5 {*Streptococcus dysagalactiae*): Positive for specific *tuf* gene.



Photograph (2): Agarose gel electrophoresis of PCR amplified products using *mecA* primers for detection of Methicillin-Resistant *S. aureus* "MRSA". Lane M: 100 bp ladder as molecular DNA marker. Lane 1: Control negative for MRSA gene. Lane 2: Control positive for MRSA gene. Lane 3: Positive tested strain for MRSA gene.

4. DISCUSSION

In the present study as shown in Table (1), the prevalence rate of bacterial isolates from milk of clinically mastitic cows in different areas at Menofya Governorate revealed an incidence of streptococcus agalactia, streptococcus dysagalactia and staphylococcus aureus 11.5%, 2.3 %, and 10% respectively. The obtained results was agreement with the results obtained by Mungube et al., (2005) and Halasa et al., (2009). *Streptococcus agalactiae* is a highly infectious bovine mastitis pathogen that can rapidly spread throughout a heard from a single infected animal. So that, early diagnosis of the infection in a heard is important for effective control. It is important to identify the presence of streptococcus agalactiae in a heard with the appearance of the first infected animal (Keef 1997) Bovine mastitis caused by *staphylococcus aureus* inflicts a numerous economic loss on dairy farms and is characterized by persistent and contagious in nature. Some strains of *staphylococcus aureus* demonstrate antibiotic resistance and may persist for longer period without overt symptoms (Aires et al., 2007 and Tenover et al., 1994). Molecular detection of *streptococcus agalactiae* by PCR in this

study was supported by Daniel et al., (1997) who showed that exact identification of pathogens is important for mastitis control and epidemiological studies. Moreover, development of molecular biological techniques such PCR may be useful significantly. The isolated *streptococcus agalactiae* were further confirmed by PCR as molecular technique which characterized by accuracy and rapidity in comparison to conventional biochemical test (Odierno et al., 2006 and Nithin et al., 2012).

5. CONCLUSION

PCR tests based on composite milk samples collected during routine milk recordings can be used as valuable tools in detection and control mastitis pathogens due to their high sensitivity ,specificity, rapidity ,ease of automation and suitability for all types of milk samples (fresh or preserved)

6. REFERENCES

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