Abstract:

Bovine mastitis is a serious problem in the dairy herds all over the world. In this study 105 mastitic milk samples were collected from small holder’s cows for bacteriological and molecular diagnosis. The prevalence of clinical and subclinical mastitis were 20.5% and 32% respectively which were detected by clinical examination and California Mastitis Test respectively. Bacteriological results revealed that Staphylococcus aureus was the most common isolated bacteria from both clinical and subclinical mastitis. Streptococcus species, Pseudomonas species, E. coli and Enterobacter species were also isolated. Application of multiplex PCR was effective in identification of bacteria causing mastitis directly from milk samples and from extracted DNA of bacteria.

Keywords: Mastitis, Prevalence, Staphylococcus aureus, Multiplex PCR.

Introduction

Mastitis is a global problem in dairy herds as it adversely affects animal health, and milk production (Sharma et al., 2012). High losses attributed to mastitis includes reduction of milk yield, low grade of milk quality, premature culling and treatment costs (Fetrow, 2000). Wide variety of bacteria can be involved in udder infection, but the most common bacteria causing mastitis in Egypt are Staphylococcus Species, Streptococcus Species and Escherichia coli (Ahmed and Mohammed, 2008). Application of PCR for mastitis diagnosis will be helpful for rapid application of the preventive measures of the disease (Qing-Hil et al., 2008).
be specific, sensitive and rapid for detection of bacteria in raw milk which helps in and control of the infection (Yu-Ping et al., 2007).

**Material and methods**

1- **Milk samples:**
A total of 105 mastitic milk samples were collected from small holder’s cows in Menoufia province according to Edmondson and Bramely (2004). Clinical signs of acute mastitis were detected in 41 cases by clinical examination and 64 samples from subclinical cases which were detected by California Mastitis Test (CMT).

2- **Bacterial identification:** was carried out according to Cruickshank et al., (1975). through culturing onto blood agar, Baired parker agar medium, Edward's medium and MacConkey agar medium, Gram’s staining and biochemical tests.

3- **DNA Extraction** from isolated bacteria and from milk samples were preformed according to Riffon et al., (2001). DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

4- **Multiplex PCR:** the reaction mixture consists of 10 µl DNA template, 25 µl of 2X Taq master mixes, 1 µl of each primers, and 13 µl of DNA free water. The PCR was carried out with preliminary step at 95°C for 2 minutes, followed by 35 cycles consisting of: 1 minute of denaturation at 95°C, 1 minute of denaturation at 45°C, 30 seconds of denaturation at 72°C, and the final extension at 72°C for 10 minutes. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 10 µl of the products was loaded in each gel slot. A 100 bp and 100DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.
Results:
The prevalence rate of clinical mastitis in dairy cows was 20.5% depending on the clinical signs of mastitis which appeared on the animals. While the prevalence rate of subclinical mastitis depending on CMT was 32%. Bacteriological examination of milk samples collected from both clinical and subclinical cases resulted in isolation of *Staphylococcus aureus*, *Streptococcus* species, *Pseudomonas* species, *E. coli* and *Enterobacter*. Table (2) illustrated the percentage of each bacterial species in milk samples of both clinical and subclinical cases.

**Table (1): Primers: sequences and target genes for staphylococcus aureus, streptococcus species and E coli** according to Pradhan et al., (2011).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU-F</td>
<td>TTC GTA CCA GCC AGA GGT GGA</td>
<td>16s-23s rRNA</td>
</tr>
<tr>
<td>SU-R</td>
<td>TCT TCA GCG CAT CAC CAA TGC C</td>
<td>229bp</td>
</tr>
<tr>
<td>ST-F</td>
<td>GAT ACA TAG CCG ACC TGA GA</td>
<td>16s rRNA</td>
</tr>
<tr>
<td>ST-R</td>
<td>AGG GCC TAA CAC CTA GCA CT</td>
<td>561bp</td>
</tr>
<tr>
<td>ECO-F</td>
<td>TCT GCG GGA GTC TCA GGG ATG GCT G</td>
<td>(tra Tgene 313bp</td>
</tr>
<tr>
<td>ECO-R</td>
<td>GTA TTT ATG CTG GTT ACC TGT TT</td>
<td></td>
</tr>
</tbody>
</table>

**Table (2): Results of bacteriological examination of milk samples from both clinical and subclinical mastitis.**

<table>
<thead>
<tr>
<th>Table (2)</th>
<th>Clinical mastitis</th>
<th>Sub clinical mastitis</th>
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<tbody>
<tr>
<td></td>
<td>Number of isolates</td>
<td>%</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>23</td>
<td>29.11</td>
</tr>
<tr>
<td><em>Streptococcus Species</em></td>
<td>13</td>
<td>16.5</td>
</tr>
<tr>
<td><em>Pseudomonas Species</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td>1</td>
<td>1.27</td>
</tr>
<tr>
<td><em>Enterobacter species</em></td>
<td>1</td>
<td>1.27</td>
</tr>
</tbody>
</table>

Application of multiplex PCR resulted in amplification of target genes at expected sites for *Staphylococcus aureus*, *Streptococcus* species and *E.coli* (229 bp, 561 bp and 313 bp respectively) in both DNA extracted from bacterial isolates and raw milk samples fig (1,2).

**Fig (1):** Results of PCR from bacterial culture by different primers combination. Lane M: 100-bp DNA ladder, Lane 1: S.aureus, Lane 2: Streptococcus Species; Lane 3: E.coli, Lane 4: Mixture of the three isolates S.aureus, Streptococcus Species and E.coli.

**Fig (2):** Results of P.C.R. on milk samples with different primer combination. Lane M: 100-bp DNA ladder, Lane 1: S.aureus at 229 bp and Streptococcus Species at 561 bp Lane 2: S.aureus at 229 bp Streptococcus Species at 561 bp and E coli at 313 bp, Lane 3: S.aureus at 229 bp, Lane 4: E.coli at 313 bp, Lane 5: S.aureus at 229 bp and L Streptococcus Species at 561 bp.
Discussion:
Mastitis is a highly prevalent problem in dairy cattle and one of the most important threats affecting the world's dairy industry Wallenberg and Vanior (2002). The present study reported a prevalence rate of clinical mastitis of 20.5%. Nearly similar results of clinical mastitis (20.43%) were obtained by Ahmed and Mohammed (2009) in Friesian cattle. While low prevalence rates were detected in Netherlands (12.7) (Miltenburg et al., 1996) and Ethiopia (10.3%) (Delelesse 2010). Prevalence of subclinical mastitis was (32%) which is nearly similar (30% and 31.75%) to that obtained by Seddek et al., (2000) and Hussein et al., (2009) respectively. A lower prevalence rate (9.96%) was reported by Ahmed et al., (2008). On the other hand a higher prevalence rate of 75.9% was reported by Karimuribo et al., (2008). There was a large variation in the prevalence of clinical and subclinical mastitis rates which may be attributed to some managemental factors such as using of dry cow therapy, feeding patterns, heifer replacement rates, environmental condition surroundings the animals, prevalent microorganism McDougall (1999). Bacteriological examination revealed that Staphylococcus aureus is the most prevalent isolate in clinical and sub clinical (29.11%), and (21.51%) respectively. This was in agreement with El-Seedy et al., (2010) and Zeryehun et al., 2013). Streptococcus species were identified (16.5% and 20.2%) in clinical and subclinical mastitis respectively. These results agree with Zeryehun et al., (2013) as they recorded a percentage of 39.9% of Streptococcus species isolated from clinical and subclinical mastitis. A lower percentage of Streptococcus species (14.2%, and 5.5%) were recorded by Matios et al., (2009); Harini and Sumathi (2011) respectively. Six isolates of Pseudomonas Species (7.6%) were identified from subclinical mastitis case. These results agree with Kivaraia and Noordhuizen (2007) and Zeryehun et al., (2013) as they recorded 7.5% Pseudomonas aeruginosa of the total bacterial isolates. E. coli and Enterobacter aerogens were identified from both clinical and subclinical mastitis (1.27%) for each organism. A higher percentage of (5.9% and 18.7%) were recorded by Anakalo and Gathoni (2004) and Ahmed and Mohammed (2009). These findings are supported by Bradley and Green (1997) who stated that Coli form particularly E. coli, Enterobacter aerogenese were the chief organisms that caused environmental mastitis. Application of multiplex PCR on bacterial isolates showed Staphylococcus aureus at 229 bp, Streptococcus Species at 561bp and E. coli
at 313 bp. These results are in agreement with (Alaa et al., 2008 and Phuektes et al., 2001). Also application of multiplex PCR on milk samples directly without the need for culture step showed Staphylococcus aureus, Streptococcus species and E coli were detected at 229 bp, 561 bp and 313 bp) respectively. The obtained results are in agreement with Yamagishi et al., (2007) and Amin et al., (2011).

References:


Pradhan, P.; Gopinath, S.M.; Reddy, G.R.; Dechamma, H. and Suryanarayana, V.V.S. (2011):


