Bacteriological and Molecular Studies on some Bacteria Isolated From Mastitic Cattle and Humans Contact

Ashraf A. Abd EL-Tawab, Fatma, I. El-Hofy, Khalid I. El-ekhnawey and Fawzia A. El-Shenawey

1Bacteriology, Immunology and Mycology Department Faculty of Veterinary Medicine, Benha University
2Animal Health Research Institute, Dokki, Egypt
3Animal Health Research Institute, Tanta, Egypt

ABSTRACT

This study was done on a total of 92 mastitic milk samples (50 clinical, 42 sub-clinical) and 40 hand swabs from contact humans were collected from different dairy farms at Gharbia governorate. The collected samples were examined bacteriologically to isolate mastitis pathogens (Staph. aureus, Strept. agalactiae, S. dysgalactiae and Strept. uberis). From clinical mastitic samples, six isolates were S. aureus (12%) and one isolate was (2%) S. dysgalactiae. Among sub clinical mastitic milk samples two isolates were S. aureus (4.6%) and one isolate (2.3%) S. agalactiae. While S. uberis were not detected. From contact human hand swabs both S. aureus and Streptococcus species were not detected. Antibiotic sensitivity test revealed that all bacterial isolates were highly sensitive to enrofloxacin, ciprofloxacin, sulpha trimethoprim and gentamicin respectively, while all isolates were resistant to penicillin followed by amoxicillin/Clavulanic acid. Two isolates of S. aureus were screened for detection of enterotoxin genes (Sea, Seb, Sec, Sed and See) by multiplex PCR. Only Sed gene was detected in one isolate. Cfb gene (CAMP factor) and hyl (hyluronidase) gene were detected in S. agalactiae. mig (surface-expressed mig protein) gene was detected in S. dysgalactiae.

Key words: Mastitic bacteria, Cattle, Humans contact

1. INTRODUCTION

Mastitis is an important disease that limits dairy production. The disease should be studied as it causes financial loss as a result of reduced milk yield, discarded milk following antibiotic therapy, veterinary expense and culling of mastitic cows (Radostitis et al., 2007). It is primarily resulting from an invasion of mammary tissues by pathogenic microorganisms through the teat canal resulting in physical, chemical, pathological changes in glandular tissues and milk (Quinn et al., 2002 and Radostitis, 2007) Many infectious agents are responsible in causing the disease in dairy animals as bacterial agents like Staphylococcus spp., Streptococcus spp., Escherichia coli, Corynebacterium spp., Klebsiella spp., Psudomonas spp., Mycoplasmal agents, fungal agents, viral agents are responsible for the disease (Radostits et al., 1995). Mastitis can occur in a clinical and sub clinical form, the latter is commonly occurring in most
herds (Gruet et al., 2001 and Awale et al., 2012). The controls of mastitis in dairy herds are accomplished in part with the aid of antibiotics therapy (NMC, 1999). *Staphylococcus aureus* is a versatile pathogen responsible for a variety of infections in humans and animals (Hata et al., 2008). The production of enterotoxins is particularly significant from a public health standpoint as the ingestion of preformed toxins is a major cause of food poisoning worldwide (Le Loir et al., 2003 and Srinivasan et al., 2006).

*S. agalactiae* is a highly contagious agent and commonly found in the mammary gland of cattle (Fonseca and Santos.,2000) and usually associated with acute clinical -mastitis and persistent subclinical infections (Hillerton et al., 2004). The molecular tests on *S. agalactiae* indicated the presence of virulence genes as *fbsA* ( encoding fibrinogen-binding protein),and *hyl* (encodes hyalurinidase enzyme ) which play a role in pathogenesis of *S. agalactiae* (Arbini et al., 2016).

The *fbsA* gene is responsible for encoding the protein *fbsA*, which allows the binding of *S. agalactiae* to fibrinogen, soluble or mobilized from extracellular matrix of the host organism (Sukhnanand et al., 2005). The adherence of *S. agalactiae* to host tissues is important in the early infection process (Frost et al.,1977 and Rosini et al 2006), and recent studies have shown that the protein *fbsA* also has platelet function and may cause other problems during infection (Pietrocola et al.,2005), but may also be involved escape mechanism in the immune system, preventing opsonization by macrophages and neutrophils (Sukhnanand et al., 2005).

The gene is responsible for *hlyB* protein called hyaluronatelyase [hlyB], which is very important for the pathogenesis of *S. agalactiae* (Glazer et al.,2002). This protein belongs to a special group of enzymes called hyaluronidase that responsible for the degradation of polysaccharides such as chondroitin, chondroitin sulfate, and especially the N acetyl glucosamine, which is part of the composition of hyaluronic acid facilitating the spread of *S. agalactiae* during infection (Akhtar et al., 2006).

Among the environmental streptococci, *S. dysgalactiae* is one of the most prevalent, which may infect mammary glands as favorable conditions are present (Todhunter et al., 1995). It can produce a surface-expressed M-like proteins called *mig*, which promote dissemination of the organism into host tissue (calvinho et al., 1998).

So, the current study aimed to isolate and identify some bacteria from mastitic milk and human contact and to detect some of their virulence genes using biochemical tests and PCR respectively.

2. MATERIAL AND METHODS:

2.I. Sampling: a total of 92 mastitic milk samples (50 clinical, 42 sub-clinical) and 40 hand swabs from contact humans were collected from different dairy farms at Gharbia governorate.  

A. Milk samples:  
Mastitis milk samples were collected aseptically into screw capped bottles and kept at 4°C until microbiological examination.

Twenty five ml from each sample were homogenized with 225 ml of buffered peptone water (BPW) for pre-enrichment and incubated at 37°C for 24 h (Addis et al., 2011).

B. Contact human hand swabs:  
Moistened sterile swabs were rolled over the palm of hands, finger tips , nails and area between fingers of human contacts. Each swab was inserted in tubes containing BPW.
for pre-enrichment.

2.2. Bacterial isolation by cultivation: A loopful from the pre-enriched culture homogenate in BPW was streaked onto the surface of Baird Parker agar, mannitol salt agar and Edward's medium. The inoculated plates were incubated at 37°C for 24 to 48 hours then examined for bacteriological growth. Suspected colonies which appeared on different media were sub cultured, purified, and preserved in semisolid agar for further identification. Bacterial colonies were identified morphologically and microscopically using Gram stain as well as biochemically using methods described by Koneman et al., (1988) and Quinn et al., (2002).

2.3. Antimicrobial susceptibility testing: It was done according to Quinn et al. (1994) and Winn et al. (2006): The obtained bacterial isolates were tested in vitro for their susceptibility to the following antimicrobial discs: enero floxacin (Enr10), ciprofloxacin (Cip5), penicillin (P10), amoxicillin/Clavulanic acid (Amc10), oxytetracyclin (OT30), gentamicin (Gen30) and sulpha trimethoprim (Sxt25).

2.4. Extraction of bacterial DNA: DNA was purified according to QIAamp DNA mini kit instructions.

2.5. Multiplex PCR for identification of Streptococcus species:

A purified DNA of S. agalactiae, S. dysgalactiae and S. uberis isolates was subjected to a multiplex PCR for the identification according to (Raemy et al., 2013) as shown in the table(1).

2.6. Multiplex PCR for detection of Staph. aureus enterotoxins:

Purified DNA of Staph. aureus isolates was subjected to a multiplex PCR for the identification of enterotoxins according to (Mehrotra et al., 2000), as shown in the table (2) and agarose gel electrophoreses according to (Sambrook et al., 1989) with agarose gel (1.5 g).

Cycling conditions of the primers during PCR : Temperature and time conditions of the two primers during PCR are shown in table (3) according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

3. RESULTS:

Incidence of bacterial species: from clinical mastitic milk samples a total of six isolates of S. aureus (12%) and one isolate (2%) of S. dysgalactiae were isolated. Among sub clinical mastitic milk samples two isolates of S. aureus (4.6%) and one isolate (2.3%) of S. agalactiae. While S. uberis were not detected. In contact human hand swabs no S. aureus and no Streptococcus species were detected.

Antibiotic sensitivity test results:

Antibiotic sensitivity determination revealed that all bacterial isolates were susceptible to enero floxacin (100%), ciprofloxacin (90%), sulpha/trimethoprim and gentamicin (70%). Moderate sensitivity to oxytetracyclin (40%). On the other hand all isolates were resistant to penicillin followed by amoxicillin/Clavulanic acid.

Detection of enterotoxin genes in Staph. aureus by multiplex PCR: Two isolates of S. aureus were screened randomly for detection of enterotoxin virulence genes by multiplex PCR and the result revealed that one isolate contain only Sed gene.

Detection of virulence genes in S. agalactiae and S. dysgalactiae by PCR: cfb gene (CAMP factor) and hyl (hyaluronidase) gene were detected in S. agalactiae. mig (surface-expressed mig protein) gene was detected in S. dysgalactiae.
Table (1): Designing of primers used for *Streptococcus* species identification:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>sequence Primer</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. agalactiae</em> cfb</td>
<td>F TTTACCAGCTGTATTAGAAGTA</td>
<td>153 bp</td>
<td>Ke <em>et al.</em>, 2000</td>
</tr>
<tr>
<td></td>
<td>R GTTCCCTGAACATTATCTTTTGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em> hyl</td>
<td>F CATACCTTAACAAAGATATATAACA</td>
<td>950 bp</td>
<td>Krishnaveni <em>et al.</em>, 2014</td>
</tr>
<tr>
<td></td>
<td>R AGATTTTTTGAAGAATGAGAAGGTTTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. dysgalactiae</em> mig</td>
<td>F CGTTTTTAGTTCGGGAGCA</td>
<td>188 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R TGCTTCAATTAGATCTGCTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (2): Designing of primers used for detection *Staph. aureus* enterotoxins:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea</td>
<td>F GGTTATCAATGTGCGGGTG</td>
<td>102 bp</td>
<td>Mehrotra <em>et al.</em>, 2000</td>
</tr>
<tr>
<td></td>
<td>R CGGCACCTTTTCTTCTCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seb</td>
<td>F GTATGGTGTTGTAAGTGC</td>
<td>164 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CCAATAGTGACGAGGTTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sec</td>
<td>F AGATGAAGTAGTTGATGTATGG</td>
<td>451 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CACACTTTAGAATCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sed</td>
<td>F CCAATAATAGGAAAATAAAAG</td>
<td>278 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R ATGTTATTTTTTCTGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>See</td>
<td>F AGGTTTTTTCAGGTCATCC</td>
<td>209 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CTTTTTTCTTTCGGTCAATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (3): Cycling conditions of the primers during cPCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> enterotoxin</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>50°C 45 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>Streptococcus virulence genes</td>
<td>95°C 15 min.</td>
<td>94°C 60 sec</td>
<td>54.6°C 60 sec</td>
<td>72°C 60 sec</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
</tbody>
</table>
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Figure (1): Gel electrophoresis pattern of multiplex PCR for *Streptococcus* species identification: Lane (2): amplification of *sklA3* gene of *S. agalactiae* at 487 bp. Lane (12): amplification of *16S* RNA gene of *S. dysgalactiae* at 279 bp.

Figure (2): Gel electrophoresis pattern of multiplex PCR for detection of enterotoxigenes of *S. aureus*: Lane (1) positive amplification of *Se* gene at 278 bp, L: ladder from 100 bp to 600 bp, Pos: positive control, Neg: Negative control.
Figure (3): Gel electrophoresis pattern for detection of some virulence genes of *S. agalactiae* and *S. dysgalactiae*: At right lane 1 positive amplification of *hyl* gene at 950 bp, lane 2 positive amplification of *cfb* gene at 153 bp. At left lane 1 positive amplification of 16S rRNA gene, Lane 2 positive amplification of *mig* gene at 188 bp.
4. DISCUSSION:
Regarding to obtained data *Staph. aureus* was the major pathogen causing clinical mastitis with incidence of (12%). Similar result of (13.5%) from clinical mastitis was detected previously by Deif , (2011). Higher incidence of *Staph. aureus* (28.32%) was detected by Ali et al. (2011), (53.4%) by Alekish et al. (2013), and (43.3%) by Duguma et al. (2014). Lower *Staph. Aureus* isolation rate (6.7%) from clinical mastitis than the current study was previously reported by Sargeant et al. (1998), and (10%) by El-Dahshan and Nada (2015). In the current study *S. aureus* was isolated with percentage of (4.6%) from sub-clinical mastitis. Higher incidence of *Staph. aureus* isolates from sub-clinical mastitis with percentage of (29%) was recorded by Calderon and Virginia (2008) and (52.2%) by Abdel-Rady and Sayed (2009), 35.71% by Abeer et al. (2010) and (26.08%) by Hande et al. (2015). Higher incidence of *S. aureus* may be attributed to the fact that the principal reservoirs of *S. aureus* are the skin of the udder and milk of infected gland also *S. aureus* is a contagious organism with capacity to penetrate into the tissue producing deep seated foci (Ranjan et al., 2011).

*Staphylococcus aureus* is the most important and prevalent contagious mammary pathogen. It causes clinical and subclinical intra mammary infection with serious economic loss and herd management problems in dairy cows (Dego et al., 2002). Nearly 95% of staphylococcal food poisoning are caused by SE types from SEA to SEE by (Tamarapu et al. 2001), SEA is considered the most commonly involved enterotoxin (Balaban and Rasooly, 2000). Such toxins are the more resistant and the ingestion of at least 1 gram of enterotoxin per 100 grams of food is enough to induce food poisoning (Tranter, 1996; Cremonsei et al., 2005). Therefore, the determination of SEs producing strains in food is important with respect to assessing public health risks (Aydin et al., 2011).

In the present study two isolates of *S. aureus* were examined randomly for detection of enterotoxin virulence genes (*Sea, Seb, Sec, Sed and See*) by multiplex PCR, only *Sed* gene was detected . this result agreed with the results obtained by Elsayed et al. (2015) who detected enterotoxine type D on *Staphylococcus aureus* isolated from clinical and subclinical mastitis. While Abeer et al.(2010) detected *Sea* and *Seb* genes in bovine milk samples. On the other hand Mohamed et al. (2016) reported that none of *Staph. aureus* strains which isolated from mastitic milk samples produce enterotoxins A, B, C, D or E. Milkers’ hands are considered as an initial point of contamination with *Staph. aureus* in dairy farms (Olivindo et al., 2009). Nearly, 30-50% of humans carry *Staph. aureus* and one third to one half of the organisms have been shown to be
enterotoxigenic (Bergdoll, 1989).

Regarding to isolation of *Staph. aureus* from contact human hand swabs in the present study, it was not isolated from contact human hand swabs. In other studies, the isolation rate of *Staph. aureus* from milkers’ hand swabs were previously reported by Lee et al. (2012) with percentage of 3.3% in Brazil and 45.9% by Adesiyun et al. (1998) in Trinidad. Moreover, an isolation rate of 44.1% was reported in skin swabs of dairy workers in Aswan, Egypt by Abdel-All et al. (2010) and was isolated with percentage of 10% from milkers’ hand swabs by El-Gedawy et al. (2014) in Sharkia, Egypt.

*Streptococcus* species as *S. agalactiae*, *S. dysgalactiae* and *S. uberis* have been reported as 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., 1992) and Picard et al. (2004).

In the present study it is clear that isolation of *Streptococci* has been limited by both species of *S. agalactiae* with percentage of (2.3%) from sub clinical mastitis and *S. dysgalactiae* with percentage of (2%) from clinical mastitis, while were not isolated from contact human hand swabs. These results were nearly similar to results of Heba (2011) who has isolated *S. agalactiae* with percent (3.1%) from clinical mastitic cases and (2%) from sub clinical mastitic cases and *S. dysgalactiae* with (1.2%) from clinical mastitic cases and (1.3%) from sub clinical mastitic cases. Also lower incidence of *S. agalactiae* were recovered by El-Zubeir et al. (2006), at rate of 0.83%. Lower isolation rate of *S. dysgalactiae* with (2.5%) and (4%) was recorded by Balakrishnan et al. (2004) and Turutoglu et al. (1995) respectively. On the other hand, higher incidences of *S. agalactiae* isolated from mastitic cows were recovered by Borkowoska et al. (2006) and Bi et al. (2016) with isolation rates of 84.8% and 92.2%, respectively. Higher isolation rate of *S. dysgalactiae* with 14%, 17% and 72.3% was recorded by Moges et al. (2011), El Jakee et al. (2013) and Bi et al. (2016) respectively.

*S. agalactiae* is an obligate organism of the epithelium and tissue of the mammary gland. It can be eradicated from dairy herds, through detection and segregation of infected cows, using hygienic milking and intra mammary infusion of antimicrobial agents (Schalm et al., 1971); Gyles and Thoen (1993). Some virulence factors, including fibrinogen binding protein (*fnb*), hyaluronatelyase, and CAMP factor (*cfb*) are responsible for *S. agalactiae* infections (Franken et al., 2001) and Beckmann et al., 2002). In the present study, PCR detection of cfb gene encoding for CAMP factor and *hyl* gene encoding for hyaluronidase gene of *S. agalactiae* revealed that, cfb gene was amplified at 153 bp. These results are in accordance with the results of Krishnaveni et al. (2014) and El-Gedawy et al. (2014) who
have been reported that cfb gene was detected in all S. agalactiae isolates. hyl gene was detected and amplified at 950 bp. Other study by Ayman et al. (2015) reported that hyl gene was detected in S. agalactiae isolated from milk samples with (81.39%), while Clarisse (2011) and Abdel-Tawab et al. (2017) reported that (38.8%) and (25%) respectively of S. agalactiae isolates contain hyl gene. Also S. dysgalactiae able to produce M-like protein called mig which involved in resisting phagocytosis by bovine neutrophils and spread of infection Song et al.,(2001). In the current study mig gene of S. dysgalactiae was detected and amplified at 188 bp in the examined isolate. Our finding agreed with the finding of Abdel-Tawab et al. (2017) who recorded that mig gene of S. dysgalactiae was detected in the all examined isolates of S. dysgalactiae. However, Ibrahim et al. (2016) stated that mig gene was detected with percentage of (77.8%) of the examined isolates.

Antibiotic sensitivity determination revealed that all bacterial isolates (S. aureus, and Streptococcus spp) were fully susceptible to enerofloxacin (100%), followed by ciprofloxacin (90%), sulpha/trimethoprim and gentamicin (70%). While moderate sensitivity to oxytetracyclin (40%). On the other hand all isolates were fully resistant to penicillin and amoxicillin/Clavulanic acid. These results go in parallel with the results of Al-ekish et al. (2013), Chandrasekaran et al., (2014), Idriss et al. (2014), Yasin et al. (2016) and Tavakoli and Pourtaghi (2017).

5. CONCLUSION:
Detection of some bacteria as S. aureus and Streptococcus species from mastitic milk of cattle and contact human was found to be an important point for animal health and contact humans. Multiplex PCR can be used as rapid and accurate method for detection of Streptococcus mastitis and virulence genes of Streptococcus and Staphylococcus aureus. Enerofloxacin and ciprofloxacin were the most effective antibiotics on treatment of cattle mastitis.
6. REFERENCES:


