Methicillin-Resistant Staphylococci in Mastitic Animals in Egypt

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Abstract: Twenty six staphylococci strains were isolated from 40 milk samples with a prevalence 11(16) 68.75% and 15(24) 62.5% of clinical and sub-clinical mastitis cases respectively. The samples were collected from (cows, buffaloes and goats). All the isolates were fully identified phenotypically to coagulase positive and negative staphylococci. Antibiotic sensitivity test was carried out by using (10) antibiotic disks (Oxoid) in vitro against (26) staphylococci strains.Resistance against pencillinG, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin showed with an incidence 97.00%, resistance against ciprofloxacin with an incidence 92.00%, sulph/trimetho and amoxy/fluclox with an incidence 94.00%. According to PCR results on (10) staphylococci strains, all of them carried 16SrRNA at 228bp. Three strains carried mecA gene at 279bp and (5) strains carried mecA gene at 147bp.

Key words: MRSA • Coagulate Positive • Coagulate Negative Staphylococci And PCR

INTRODUCTION

Staphylococcus aureus represents an important etiologic agent Causing mastitis in cows, goats and sheep [1]. On the other side, Coagulase-negative staphylococci (CNS) are considered emerging agents of subclinical or mild clinical mastitis [2]. Multi-drug resistance (MDR) S. aureus and CNS were isolated from mastitic cases [3]. MRSA disseminates intra-mammary that often produces incurable severe intra-herd infections [3].

Methicillin-resistant Staphylococcus aureus (MRSA) is an important pathogen in human medicine [4]. MRSA strains acquired gene gives them resistance to methicillin and essentially all other beta-lactam antibiotics (multi-drug resistant) such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, which are often used in the treatment of mastitis [5]. MRSA strains show pathogenic and epidemiologic characteristics via mutation, clonal evolution and horizontal gene transfer [6, 7]. These evolutionary processes enhance pathogenicity and antimicrobial-resistant properties of S. aureus strains [8].

Resistance to methicillin (and β-lactams) is associated with the mecA gene, which codes for penicillin-binding protein 2a (PBP2a), a cell wall synthetic protein with low affinity in binding to β-lactams [9]. The mec genes are embedded in large mobile elements called staphylococcal cassette-chromosome-mec (SCCmec) [10]. Methicillin-resistant coagulase-negative staphylococci (MR-CoNS) generally carry a vast variety of SCCmec elements and are currently considered to be the likely reservoir of the different types of mecA gene in MRSA [11].

The aim of the study is isolation and identification of methicillin-resistant staphylococci strains either coagulase positive or negative through phenotypic and genotypic characters from mastitic farm animals (cows, buffaloes and goats) in Egypt.

MATERIALS AND METHODS

Sample Collection: A total of (40) milk samples were collected from cattle (no=20), buffaloes (no=15) and goats (no=5). All samples were collected in clean and sterile tubes. Milk samples were examined by California Mastitis Test (CMT), then transported to the laboratory on ice packs without delay.
Table 1: primers sequences for the *Staphylococcus* genus (16SrRNA), thermostable nuclease gene for *S. aureus* (*nucl*) and methicillin resistance gene (*mecA*) and product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 16SrRNA | F.5’GTAGGTGCAAGCGTTAT3’  
R.5’CGCACATCAGCAGTCAG3’ | 228bp | Monday and Bohach. 1999[17]. |
| *nucl* | F.5’GCGATTGATGGTGATACGGTT3’  
R.5’AGCCAAGCCTTGACGAAACTAAAGC3’ | 279 bp | Brakstad et al., 1992[18]. |
| *mecA* | F.5’GTGAAGATATACCAAGTGATT3’  
R.5’ATGCGCTATAGATTGAAAGGAT3’ | 147 bp | Zhang et al., 2005[19]. |

**Isolation and Identification of *Staphylococci***: About 10 µL of collected milk samples was spread over mannitol salt and sheep blood agar plates then incubated at 37°C for 24 h. Suspected colonies were examined by Gram's stain according to Cruickshank et al., [12]. Biochemical identification was carried out according to Collee et al. [13] and CDC [14] that including: catalase, coagulase, gelatin liquefaction and sugar fermentation for glucose, maltose, lactose and sucrose.

**Antibiotic Sensitivity Test**: Twenty-six *Staphylococci* strains were examined *in vitro* against (10) different antibiotics. It was carried out by using agar diffusion antibiotic sensitivity according to Beaney et al., [15]. Interpretation was carried out according to NCCLS, [16]. Antibiotic discs were obtained from Oxoid which including: penicillin-G (10 µunits), erythromycin (15 µg/ml), gentamicin (10 µg/ml), ciprofloxacin (5 µg/ml), tetracycline (30 µg/ml), sulphita/trimetho (23.75+1.25 µg/ml), amikacin (30µg/ml), amoxyl/fluclox (25 µg/ml) and oxacillin (1 µg/ml) and cefoxitine (30 µg/ml).

**DNA Extraction and PCR Amplification**: Genomic DNA of staphylococci strains were extracted by using an extraction kit (QIA amp mini kit,Qiagen). Specific oligonucleotide primers for the [*Staphylococcus* genus (16SrRNA), thermostable nuclease gene for *S. aureus* (*nucl*) and methicillin resistance gene (*mecA*)] genes were used. The amplification conditions included initial denaturation step at 94 °C for 4 min and 35 cycles of denaturation at 94 °C for 60 sec, primer annealing at 55 °C for 60sec, extension at 72 °C for 60 s and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed. Primers sequences and amplified product size were showed in table (1).

**RESULTS**

All milk samples were collected and classified as clinical or subclinical mastitic cases according to the results of California mastitis test. A total of (26) *staphylococci* strains isolated were from (40) mastitic milk samples from cows, buffaloes and goats. The suspected colonies were circular, smooth and glistening. Gram's stain revealed Gram positive, non-spor forming cocci, arranged in grapes or irregular clusters. The results of biochemical tests proved that suspected colonies to be *staphylococci* spp.

The numbers of samples and type of mastitis were illustrated in Table (2).

Antibiotic sensitivity test was carried out using (10) antibiotic disks (Oxoid) *in vitro* against (26) *Staphylococci* strains showed resistance against penicillin–G, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin with an incidence 97%, resistance against ciprofloxacin with an incidence 92%. sulph/trimethoamoxyl/fluclox with an prevalence 94%.

**Table 2: Number of samples and type of mastitis cases**

<table>
<thead>
<tr>
<th>Host</th>
<th>No. of samples</th>
<th>Clinical</th>
<th>Subclinical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>20</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Buffalo</td>
<td>15</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Goats</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>16</td>
<td>24</td>
</tr>
</tbody>
</table>

Coagulase test was used to identify the isolates to coagulate positive (CPS) and CNS as showed in table (3).
Table 3: Number and incidence of coagulase positive (CPS) and coagulase negative staphylococci (CNS)

<table>
<thead>
<tr>
<th>Host</th>
<th>Clinical</th>
<th>Sub-clinical</th>
<th>No.</th>
<th>% (CPS)</th>
<th>Clinical</th>
<th>Sub-clinical</th>
<th>No.</th>
<th>% (CNS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>3(8)</td>
<td>3(12)</td>
<td>2</td>
<td>37.5%</td>
<td>2 (8)</td>
<td>5(12)</td>
<td>2</td>
<td>41.66%</td>
</tr>
<tr>
<td>Buffalo</td>
<td>1(6)</td>
<td>3(9)</td>
<td>2</td>
<td>16%</td>
<td>2(6)</td>
<td>2(9)</td>
<td>2</td>
<td>22.22%</td>
</tr>
<tr>
<td>Goat</td>
<td>2(2)</td>
<td></td>
<td></td>
<td>100%</td>
<td>1(2)</td>
<td>2(3)</td>
<td></td>
<td>66.66%</td>
</tr>
</tbody>
</table>

Total *Staphylococci* strains No. and %

<table>
<thead>
<tr>
<th>Host</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td>11</td>
<td>68.75%</td>
</tr>
<tr>
<td>Sub-clinical</td>
<td>15(24)</td>
<td>62.5%</td>
</tr>
</tbody>
</table>

Table 4: Relationship between (CPS) and (CNS) and 16SrRNA, nuc and mecA genes

<table>
<thead>
<tr>
<th>Strain host</th>
<th>CPS</th>
<th>CNS</th>
<th>16SrRNA</th>
<th>nuc</th>
<th>mecA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Buffalo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Buffalo</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Buffalo</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Cow</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Cow</td>
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<tr>
<td>Cow</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

Photo 1: Amplified PCR product of (16SrRNA) gene at (228 bp). Lane M: 100bp ladder, Lane 1-10 positive to *genus staphylococci*.

A total of (10) staphylococci strains which showed 100% resistance to the previous antibiotics were chosen for PCR. Among (10) staphylococci strains, (3) strains (cows, buffaloes and goats) are (CNS) and the rest of strains are (CPS). The result of detection to (16SrRNA, nuc and mecA) genes and their relationship with CPS and CNS were illustrated in table (4).

**DISCUSSION**

Mastitis faces dairy production industry. Economic significances of the mastitis are related to the reduced milk quantity and quality, veterinary expenses and milk loss due to the antibiotic withdrawal time post treatment [20].
Staphylococci are common etiological agents of mastitis [21]. In our study, 26 staphylococci strains [11 (16) 68.75% and 15 (24) 62.5%] were isolated from clinical and sub-clinical mastitis milk samples respectively, this isolation prevalence nearly agreed with [22] Asfour and Darwish who isolated Staphylococci with prevalence of 52% and 67% from clinical and sub-clinical bovine mastitis milk samples respectively.

The number and prevalence of CPS strains in clinical and subclinical mastitis cases in cows were 37.5 and 25% while CNS strains in clinical and subclinical mastitis cases were 25 and 41.6% respectively. In buffaloes, CPS strains were 16 and 33.33% and CNS strains were 33.33 and 22.22% in clinical and sub-clinical mastitis cases respectively.

In goats, CPS strains in clinical and subclinical mastitis were 100 and 0 %, while CNS strains were 50 and 66.66% in clinical and subclinical mastitis cases respectively. According to the previous results, the incidence of isolation of CNS is higher than CPS in sub-clinical mastitis cases of cows' and goats' milk, these result was in harmony with Taponen and Pyorala [2] who stated that CNS became the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens. CNS occasionally causes sub-clinical or clinical mastitis [23].
In addition it elevates somatic cell count in infected quarters [24], hurts udder tissue, decreases milk quality and quantity [25] so identification of CNS species is essential to determine their pathogenicity and to develop management practices to prevent mastitis.

The results of antibiotics sensitivity showed that staphylococci strains were resistant to pencillin-G, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin with 97.00%; ciprofloxacine with 92%; and sulph/trimetho and amox/fluclox with 94%. This finding was agreed with Abd El-Moez et al. [26] who reported that staphylococci strains resistance against cefotaxime, sulph/trimetho and amox/fluclox was 94.40%. Resistance against pencillin–G, erythromycin, gentamycin, tetracycline and amickacin was 89.00%. Resistance against ciprofloxacine was 83.30%. These results mentioned that our study staphylococci strains are multidrug-resistant and confirmed to be methicillin-resistant staphylococci (MRS), Karska-Wysocki et al., [27] explained that MRSA is a multidrug-resistant microorganism. Attention with MRSA is very important because infections caused by meticillin-resistant staphylococci (MRS) are more difficult to treat and may pose a public health risk [28]. Some MRSA isolates from bovine mastitis are thought to be bovine and some of human origin, clonal transmission between farmers and dairy cows has been shown to occur, Haenni et al. [29].

Conventional identification of MRSA requires between 24-48 hours after sampling, in addition to that accreditation on identification of MRSA by antibiotic susceptibility testing alone isn't enough so rapid and sensitive method of identification as PCR for detection of mec-A gene that codes for penicillin-binding protein (PBP2a) or 2(PBP2) are recommended. The 16SrRNA gene was detected at (228bp) in all tested strains which confirmed to be in Staphylococcus genus, this result agreed with Asfour and Darwish [22]. Out of (3) strains of (10) were carried nuc gene at (279bp) which confirmed to be Staphylococcus aureus. Five isolates were confirmed to carry mec Ageneat (147bp) which responsible for β-lactams resistance.

In our study all staphylococci strains used in PCR were oxacillin resistant but (5) strains only carried mecA gene, this can explained by presence of other β-lactam resistance gene which is blaZ gene that can affect the expression of mecA gene [30]. This result also agreed with Olsen et al. [31] who stated that there are two genetic mechanisms are associated with resistance to the β-lactam class of antibiotics in Staphylococcus spp, the most important is production of β-lactamase that may bemediated by the blaZ gene and mec Agene. On the other side, disk diffusion test gives false positive and negative results due to variation in inoculums size and growth condition [32].

There are (2) strains carried both nuc and mec Agenes, this finding stated that Meticillin-resistant Staphylococcus aureus (MRSA) were detected with an incidence (2/5) 40%, on the other side, (3) strains CPS other than S. aureus were carried mec Agene with an incidence (3/5) 60%.

This result was agreed with Park et al.[33] who stated that CPS other than S. aureus was classified as contagious pathogens infecting milk samples. While S. aureus is the most common coagulase-positive staphylococcus isolated in the clinical laboratory, S. intermedius, S. delphini, S. schleiferi sub sp. coagulans, S. lutrae and some strains of S. hyicus are also coagulase positive and have public health important so attention to CPS other than S. aureus must be considered.

CONCLUSIONS

Coagulase negative and coagulase positive staphylococci strains other than S.aureus are multidrug resistant representing a serious problem which need more attention as S.aureus, so further studies were recommended as detection of virulence genes and sequence analysis to meticillin resistance genes which able these strains to be multiple multidrug resistance.

REFERENCES


