Bacteriological and molecular studies on toxigenic *Staphylococcus aureus* in milk and some milk products

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**Abstract**

A total of 200 random samples of milk and milk products represented by kareish cheese, yoghurt and ice-cream (50 for each) were examined microbiologically for the presence of *Staphylococcus aureus*, its enterotoxigenicity and its antibiotic sensitivity. *Staphylococcus aureus* was isolated from 8 (16%) milk samples, 15 (30%) kareish cheese, 4 (8%) yoghurt and 11 (22%) ice-cream samples. All *S. aureus* isolates exhibited clumping factor using kits for reliable latex agglutination test. The susceptibility of the isolates was determined for 12 antimicrobial drugs using disc diffusion assay. The majority of strains were susceptible to ofloxacin and ampicillin + sulbactam (100%), vancomycin and tetracycline (94.7%), norfloxacin and sulphamethoxazole + trimethoprim (89.5%), chloramphenicol (73.3%) but they were resistant to oxacillin and metronidazole (100%). Amplification of coagulase gene (*coa*) using uniplex PCR, staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*) and methicillin-resistant *S. aureus* (*mecA*) gene using multiplex PCR revealed that, 11/11(100%) of the examined samples were positive for both *coa* and *mecA* genes. While, *sea* produced by 5 (45.45%) strains, *sec* and *sed* produced by 4 (36.36%) strains and *seb* and *see* were not produced by any strains.

**Key words:** milk, *S. aureus*, enterotoxigenicity, antibiotic sensitivity, PCR.

1. **Introduction**

Milk serves as an optimum medium for propagation of various pathogenic and spoilage microorganisms (Gatti et al., 2013). Staphylococcal intoxication is considered the second or the third most common food intoxication of microbiological origin (Atanassova et al., 2001). Coagulase positive *S. aureus* is the causative agent of two thirds of food-borne disease outbreaks (Busani et al., 2005). Staphylococcal food poisoning (SFP) is one of the most prevalent causes of gastroenteritis worldwide. Symptoms of SFP have a rapid onset (2 to 6 hours) of abdominal cramps, nausea, and vomiting, sometimes followed by diarrhoea. Patients become symptomatic within 2-4 hours after ingestion of thermostable staphylococcal enterotoxins (SEs) of an approximate dose of 0.1 to 1.0 mg/kg of body weight (Stewart et al., 2005). There are serologically several distinct enterotoxins that *S. aureus* produced including staphylococcal enterotoxins (SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity (Argudín et al., 2010). Antimicrobial resistance is also of an important public health concern worldwide. Methicillin resistant *S. aureus* (MRSA) strains resistant to quinolones or multi-resistant to other antibiotics have been emerging, leaving a limited choice for their control (Mee-Marquet et al., 2004; Nejma et al., 2006). PCR assay for detection of genes for *S. aureus* was developed and proved to be specific, sensitive, and rapid method (Omoe et al., 2002; Zschock et al., 2005). As the level of contamination of both milk and its products with different food-borne pathogens constitutes serious problems for consumers, so, the purpose of this study was the evaluation of bacteriological patterns of *Staphylococcus aureus* as one of the food poisoning micro-organisms in milk and milk products.
2. MATERIAL AND METHODS

2.1. Samples collections:

A total of 200 random samples of milk and milk products including yoghurt, kareish cheese and ice-cream (50 of each) were collected from different large and small dairy plants, street vendors and dairy house in El-Sharkia and Giza Governorates.

2.2. Bacteriological examination:

2.2.1. Preparation of serial dilutions (APHA, 1992):

2.2.2. Isolation and identification of staphylococcus aureus:

Isolation on Baird-Parker medium, Blood agar medium and Milk Salt agar medium was performed according to (APHA, "American Public Health Association" 1992), morphological identification by Gram stain (Cruickshank et al., 1975), biochemical tests (Arora, 2003; Quinn et al., 2002) and serologically by latex agglutination test (Staphytect plus) (Oxoid, 1990).

2.2.3. In-Vitro anti-microbial sensitivity method (Finegold and Martin, 1982):

Using agar diffusion method.

2.3. Molecular biology technique (PCR):

2.3.1. Uniplex PCR:

For detection of staphylococcal Coagulase (coa) gene using specific oligonucleotide primers sequences for these genes with the length of amplified products at 850 bp according to Goh et al. (1992).

2.3.2. Multiplex PCR:

For detection of *S. aureus* enterotoxins genes (sea, seb, sec, sed and see) and the methicillin-resistant (mecA) genes of *S. aureus* using specific oligonucleotide primers sequences with the length of amplified products for enterotoxins at (102 bp for sea), (164 bp for seb), (451 bp for sec), (278 bp for sed) and (209 bp for see). While, the length of amplified products for methicillin-resistant (mecA) genes at different base pairs (613, 398, 280, 776, 493, 200, 881 and 325 bps) according to Mehrotra et al. (2000).

3. RESULTS

The bacteriological examination of milk and milk products (kareish cheese, yoghurt and ice-cream) samples was shown in (Table, 1). The results revealed that *S. aureus* was isolated from 38 (19%) of the examined samples represented as, 8 (16%) milk samples (1 from large scale dairy plants, 1 from small scale dairy plants, 3 from farmer houses and 3 from street vendors), 15/50 (30%) kareish cheese samples (1 from large scale dairy plants, 2 from small scale dairy plants, 7 from farmers houses and 5 from street vendors), 4 (8%) yoghurt samples (0 from large scale dairy plants, 0 from small scale dairy plants, 2 from farmers houses and 2 from street vendors) and 11 (22%) ice-cream samples (0 from large scale dairy plants, 2 from small scale dairy plants, 3 from farmers houses and 6 from street vendors). The results of in-vitro sensitivity test for the isolated *S. aureus* indicated the isolated strains were susceptible to ofloxacin and ampicillin + sulbactam (100%), vancomycin and tetracycline (94.7%), norfloxacinc and sulphamethoxazole + trimethoprim (89.5%), chloramphenicol (73.3%) (Table, 2). On the other hand, moderate sensitivity was observed to cephalothin and clindamycin with percentages of 57.9% and 42.1%, respectively. Moreover, the majority were resistant to oxacillin and metronidazole (100%). Confirmation of 11 randomly selected *S. aureus* isolates from milk and milk products using molecular PCR (Table, 3) showed that, coa genes produced by all of the examined 11 (100%) samples (Photo, 1) using uniplex PCR, mecA genes produced by all of the examined 11 (100%) samples (Photo, 2) by multiplex PCR. Multiplex PCR also used for detection of staphylococcal enterotoxin genes (sea, seb, sec, sed and see) and showed that, see genes produced by 5/11 isolates (45.45%), sec and sed produced by 4/11 isolates (36.36%). While, seb and see genes did not produced by any isolate (Photo, 3).

4. DISCUSSION

The examined samples of milk and milk products (kareish cheese, yoghurt and ice-cream) have been showed *S. aureus* in 38 (19%) of milk and milk products samples Table (1). *Staphylococcus aureus* was isolated from 8 (16%) milk samples. Nearly similar findings were postulated by Alnakip (2009); Aman and Ahmed (1996); El-Bessary (2006); El-Jakee et al. (2008); Jakeen et al. (2010); Nassar (2013) who isolated *S. aureus* from 15%, 16.66%, 17.5%, 16% from buffalo’s milk and 22.7% from cow milk, 16.7%, 19.13% and 16%, respectively. On the other hand, higher incidence was reported by El-Gendy (2015); Hammad (2004); Mohammed (2002); Ralls et al. (2008); Tondo et al. (2000); Wafy (2006) who isolated *S. aureus* from 90.4%, 64%, 80%, 60%,
Table 1. Prevalence of *S. aureus* in milk and milk products (n=50):

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>No. of samples</th>
<th>Dairy plants</th>
<th>Farmers houses</th>
<th>Street vendors</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Large scale</td>
<td>Small scale</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No./10 %*</td>
<td>No./10 %*</td>
<td>No./15 %*</td>
<td>No./15 %*</td>
<td>No./50 %**</td>
</tr>
<tr>
<td>Milk</td>
<td>50</td>
<td>1</td>
<td>10</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Kareish cheese</td>
<td>50</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>13.33</td>
</tr>
<tr>
<td>Ice-cream</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

*percentage in relation to No. of each examined samples. ** percentage in relation to total No. of each 50 examined samples.

Table 2. In-Vitro antimicrobial sensitivity test for isolated *S. aureus* (CLSI, 2014):

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>S. aureus isolates %*</th>
<th>S. aureus isolates %*</th>
<th>S. aureus isolates %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin</td>
<td>38 100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ampicillin+ Sulbactam</td>
<td>38 100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>36 94.7</td>
<td>-</td>
<td>2 5.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>36 94.7</td>
<td>2 5.3</td>
<td>-</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>34 89.5</td>
<td>-</td>
<td>4 10.5</td>
</tr>
<tr>
<td>Sulphamethoxazole-Trimethoprim</td>
<td>34 89.5</td>
<td>4 10.5</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>28 73.7</td>
<td>6 15.8</td>
<td>4 10.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>18 47.4</td>
<td>16 42.1</td>
<td>4 10.5</td>
</tr>
<tr>
<td>Amoxicillin+ Clavulinic acid</td>
<td>14 36.8</td>
<td>8 21.1</td>
<td>16 42.1</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>6 15.8</td>
<td>22 57.9</td>
<td>10 26.3</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>- -</td>
<td>- -</td>
<td>38 100</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>- -</td>
<td>- -</td>
<td>38 100</td>
</tr>
</tbody>
</table>

*Percentage in relation to total number of isolated *S. aureus*

Table 3. Incidence of coagulase positive, methicillin resistant *S. aureus* and enterotoxins in randomly selected 11 examined samples of milk and milk products by PCR:

<table>
<thead>
<tr>
<th>Examined S. aureus for</th>
<th>No. of + ve samples</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase positive</td>
<td>11</td>
<td>100%</td>
</tr>
<tr>
<td>Methicillin resistant</td>
<td>11</td>
<td>100%</td>
</tr>
<tr>
<td>Enterotoxins A</td>
<td>5</td>
<td>45.45%</td>
</tr>
<tr>
<td>Enterotoxins B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterotoxins C</td>
<td>4</td>
<td>36.36%</td>
</tr>
<tr>
<td>Enterotoxins D</td>
<td>4</td>
<td>36.36%</td>
</tr>
<tr>
<td>Enterotoxins E</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
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*Percentage in relation to total number of selected S. aureus isolates

Photos 1, 2 and 3. Agarose gel electrophoresis patterns showing: 1. UniPlex PCR for the S. aureus. 2. Multiplex PCR for methicillin. coagulase (coa) gene, resistant S. aureus (mecA) gene

Lanes 1: DNA molecular size marker (100-bp ladder). Lane 2-12: positive sample for coagulase gene and methicillin resistant gene

Multiplex PCR amplification products for the S. aureus enterotoxin genes. Lane 1: DNA molecular size marker (100-bp ladder). Lanes 3, 5, 8, 10 and 11: positive samples sea gene at 102 bp. Lanes 2, 3, 9 and 12: positive samples sec gene at 451 bp. Lanes 4, 6, 7 and 10: positive samples sed gene at 278 bp

70.4% and 80%, respectively, but relatively lower incidence was achieved by Amer et al. (2007); Demo et al. (1999); Kivaria et al. (2006); Sudershan and Ashwani (1996) who isolated S. aureus from 12.96%, 14.5%, 6.3% and 13%, respectively. Moreover, S. aureus was isolated from 15/50 (30%) kareish cheese samples. Nearly similar finding to that postulated by Abo-Donia et al. (1975); Ahmed et al. (1988); El-Shater (2010); Hassan (2003) who isolated S. aureus from 33.3%, 27.5%, 30% and 28%, respectively. On the other hand, higher incidence was reported by Awida (2009); (1999); Hassan (2008); Kolta (2011); Said and Fahmy (1991) who isolated S. aureus from 78%, 42.5%, 72%, 50% and 70%, respectively, but lower incidence was reported by Ahmed Ahmed et al. (2004); Awad Allah (2004); El-Bessary (2006) who isolated S. aureus from 10 % and 11.25 % in Kafr El-Shaikh and Gharbia Governorates, 12% and 5%, respectively. S. aureus was isolated from 4/50 (8%) of the examined yoghurt. Nearly similar finding to that postulated by El-Biaa (2011); El-Shinawy (1987) who isolated S. aureus from 10% and 12%, respectively. On the other hand, higher incidence was reported by Abdel-Fatah (2007) who isolated S. aureus in 64.44% of samples. S. aureus could not be detected in industrial processing yoghurt samples; this is in agreement with Al-Tahriri (2005). S. aureus was isolated from 11/50 (22%) ice-cream samples. Nearly similar finding to that postulated by Abdel-Fatah (2010); Kamal (2009); Masud (1989) who isolated S. aureus from 26%, 22.9% and 20%, respectively. On the other hand, higher incidence was reported by Abdel-Haleem (1995); Abo-Risha (1998); Allam (1999); Hammad (2004); Hassan (2003); Patr et al. (2007) who isolated S. aureus from 84.72%, 50%, 76%, 56.67%,55% and 44% respectively, but lower incidence was reported by Amuramjimi et al. (2008); Kock et al. (1998); Kruy et al. (2001); Little and Louvois (1999); Manzanera-Pelegrin et al. (1995) who isolated S. aureus from 2.7%, 4.3%
, 0.5%, 12.2 and 4.4%, respectively. Moreover, Caglayanlar et al. (2009); Korel et al. (2002); Maiereni et al. (1993); Sagdic et al. (2002) could not detect S. aureus in any one of the examined ice-cream samples. Moreover, the results of antibiotic sensitivity tests for the isolated S. aureus Table (2) showed that, S. aureus isolates were highly sensitive to ofloxacin and ampicillin + sulbactam (100%), vancomycin and tetracycline (94.7%), norfloxacin and sulphamethoxazole + trimethoprim (89.5%), chloramphenicol (73.3%) and they were the most proper antibiotics with the highest efficiency against isolated S. aureus. Meanwhile, they were resistant to oxacillin and metronidazole (100%). Nearly similar results of norfloxacin were recorded by Ahmed (2015); Khalil (2014). Nearly similar results of vancomycin were recorded by Nassar (2013) at which the sensitivity to vancomycin was 100%. The sensitivity to ofloxacin (65%) is also recorded by Ikeagw et al. (2008). The sensitivity to sulphamethoxazole + trimethoprim (95%) is also recorded by Nassar (2013). The sensitivity to chloramphenicol is also recorded by Centorbi et al. (1992); Rossetti (1993) at which it was detected in 94% and 100%, respectively, also Bobu et al. (2007) mentioned that chloramphenicol was the most effective antibiotics. There is no resistance was detected for ampicillin + sulbactam as recorded by Gentilini et al. (2000). The sensitivity to tetracycline is also recorded by Ahmed (2015); Dutta and Rangnekar (2008); Edward et al. (2009) at which it was detected in 100%, 70% and 85.7%, respectively. Compound oxacillin used to detect MRSA strains and it is also named ORSA (Oxacillin resistant S. aureus). This study explained that all examined S. aureus strains were resistant to oxacillin, this result come in agreement to that reported by Khalil (2014) and dis-agree to those reported by Gentilini et al. (2000); Rossetti (1993) who showed that 100% of the examined strains are susceptible to oxacillin. Our PCR results Table (3) revealed that, uniplex PCR results for coa gene showed that 11/11 (100%) of the examined isolates were positive for coa gene. Detection of coagulase gene by PCR was discussed by several authors as by Adwan et al. (2005) who reported that, out of the 100 S. aureus isolates (milk sheep origin= 52, milk cows origin=48) tested for SE-genes by PCR method, 37% were positive. None of these isolates carried more than one toxin gene. The majority of these positive toxin gene isolates 20 (54.1%) were seb positive and (Shalaby, 2012) at which the multiplex PCR test revealed that enterotoxin A found in 58.3% of S. aureus isolates, both enterotoxins B and C was found in 66.7% of the isolates and enterotoxin D was the predominant one found in 75% of the isolates. The results of multiplex PCR for methicillin resistant S. aureus showed that 11/11(100%) of the examined isolates were carrying mecA gene (MRSA). Methicillin resistant S. aureus by multiplex PCR was also detected by several authors as by Bakeet and Darwish (2014); Mulders et al. (2010); Nemati et al. (2008); Oke and Adewale (2013).

The obtained results allow concluding that milk and its products were highly contaminated with high number of S. aureus which may lead to undesirable changes that render them unfit for human consumption and indicate unpersonal hygiene and unsanitary conditions during processing and handling. Also, Antimicrobial resistance are widely spread among isolated strains and has been reported to negatively affect the treatment of its associated infections in human and animals.

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