DETECTION OF ENTEROTOXIN GENES IN STAPHYLOCOCCUS AUREUS ISOLATED FROM COW MILK

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ABSTRACT

Staph. aureus is considered one of the main causes of food poisoning and clinical infections in different hosts. So this work intended to investigate the occurrence of enterotoxin genes and antibiotics susceptibility of Staph aureus isolated from raw and local pasteurized milk. To realize this, 125 samples of raw milk and 75 samples of local pasteurized milk were obtained from supermarkets, dairy shops and farms in Sohag Province, Egypt. These samples were subjected to bacteriological examination. The result showed that 68 out of 125 raw milk samples and 37 out of 75 local pasteurized milk samples were positive for Staphylococcus spp. on mannitol salt agar. Biochemical scheme supposed that 18 isolates of raw milk samples and 8 isolates of local pasteurized milk samples were positive for staph aureus. PCR by using nuc gene specific primer, discriminated the existence of Staph aureus DNA in 15 isolates out of 18 isolates of raw milk samples, while 7 isolates out of 8 isolates of local pasteurized milk samples showed a positive bands at 267bp. Staphylococcal enterotoxin genes were detected in 53.33% and 42.86% of Staph aureus isolates from raw and local pasteurized milk, respectively. sea and sed genes were detected in most isolates of Staph aureus while none of these isolates harbored seb and see genes. sec gene could be detected only in the raw milk isolates. The antibiotics sensitivity profile of enterotoxigenic Staph aureus was showed a high percentage of resistance to penicillin and tetracycline while all isolates were fully susceptible to vancomycin.

Key words: Milk, Staph aureus, nuc gene, enterotoxins, antimicrobials.

INTRODUCTION

Milk is an imperative food because it is rich by various essential components including proteins, vitamins, and minerals also it considers a nutritive media for many microorganisms such as Staph. aureus which mainly criminalized in food poisoning cases (Pandey et al., 2014), because it can adapt to grow in various types of foods and secreting enterotoxins (Balaban and Rasooly, 2000).

The contamination of milk by Staph. aureus was occurred through the infection of the mammary gland or by bad sanitary conditions, during or after milking, and these happened by human activity who responsible for the contamination (Rehman et al., 2014).

Staph. aureus is a gram-positive microorganism, grows in a different temperatures, ranged from 7 °C to 48.5 °C. It produce a broad extracellular toxins, the antigenic-base classification of Staphylococcal enterotoxins (SEs) includes five classical types of toxins (SEA-SEE) (Riva et al., 2015). The most imperative SEs are SEA and SEB which usually more common in milk (Chiang et al., 2006).

Staphylococcal enterotoxins resists the majority of proteolytic enzymes and thus remains their action in gastrointestinal tract. They are highly heat resistant toxin (Sutejo et al., 2017), they keep their activities even after pasteurization (Rallin et al., 2008). The SEs toxins resulting in nausea, brutal vomiting, abdominal pain and occasionally diarrhea (Rosengren et al., 2013).

Staph. aureus became more complicated problem for its ability to resist different types of antibiotics. The spreading of the MRSA strains worldwide lead to high costs in terms of treatment and lead to life threatening infections (Yamamoto et al., 2013).

Routine identification of Staph. aureus usually carried out by traditional methods but these methods
were discomfit and time consuming. Furthermore, these methods lead to indistinct results. Rapid and accurate methods for identification of food borne pathogens are important for microbiological safety. In the last 10 years, unrestricted detection methods using molecular techniques, such as polymerase chain reaction (PCR) method particularly multiplex PCR was proven as one of the most suitable way for sensitive and fast detection of pathogenic bacteria in food (Shawish and Al-Humam, 2016 and Kim et al., 2017).

Considering to these facts, the existing work intended to study: The presence of SEs coding genes (sea, seb, sec, sed and see) in Staph aureus strains isolated from raw and local pasteurized milk by using multiplex-PCR and evaluation the sensitivity of these isolates to different types of antimicrobials.

MATERIALS AND METHODS

I-Milk samples:
A total of 200 cow milk samples (125 raw cow milk and 75 local pasteurized cow milk) were collected randomly from local markets, street vendors and farms in Sohag Governorate, Egypt, during the period from July to September 2017. The samples were collected in sterile plastic bags. All the samples were taken to the laboratory under refrigerate conditions where they were prepared for bacteriological examination.

II- Isolation of Staph. aureus:
One milliliter of each sample was added aseptically in a sterile test tube contain 9 ml of the nutrient broth. Inoculated test tubes were incubated at 37°C for 18 hours. A loopful from each incubated broth tube was plated on manniot salt agar by using Streaking plate method. Plates were incubated at 37°C for 24-48 hours according to (Arora, 2003). The suspected colonies were purified and then transferred to nutrient agar slopes for preservation and further identification.

III-Biochemical Identification of Staph. aureus isolates:
The identification of suspected colonies were identified according to Holt et al. (1993) this identification based on colony morphology, staining reaction and biochemical tests such as catalase, coagulase test and thermonuclease test. The isolates which pretended a positive results in pervious tests were submitted to the Voges-Proskauer to discriminate Staph. aureus (positive) from other coagulase and thermonuclease positive (negative).

IV-Genotypic identification:
1-DNA extraction:
DNA was extracted from 5 ml of a coagulase-positive Staphylococcal culture grown at 35°C (±2°C) for 16-24h in nutrient broth (oxoid). DNA was extracted by QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacture instructions.

2-Molecular confirmation of Staph aureus by using conventional PCR:
Amplification reaction were performed according to Mansour et al. (2017) with slight modification, in a final volume of 50 µl containing: 25µl PCR Mastermix (Emerald Amp GT), 1.5µl for each primer for nuc gene (Table. 1), 5µl of DNA Template and 17µl of PCR grade water. Reactions were carried out in thermal cycler (MJ Research, Inc. Watertown, MA) with the following program: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 2 min, 52°C for 1min and 72°C for 1min with a final extension at 72°C for 10 min. PCR product was alienated by electrophoresis in 1.5% agarose gel (Bioshop®, Candainc.) stained with ethidium bromide, then visualized in a UV transilluminator.

3-Detection of classical enterotoxin genes in Staph. aureus isolates by using multiplex PCR:
Multiplex PCR was performed by using the following primers for enterotoxin genes (sea, seb, sec, sed and see) (Table 1), the PCR mixture was prepared as following: 12.5 µl PCR Taq green Mastermix (Thermo), 1 µl of each primer and 1µl DNA. The final volume was adjusted to 25 µl by adding sterile PCR grade water. Amplification profile was standardized in 94°C for 5 min followed by 35 cycles of 94°C for 2 min, 52°C for 2 min and 72°C for 3 min with a final extension at 72°C for 7 min. PCR products were separated by gel electrophoresis (1.5% agarose gel stained with ethidium bromide) then were visualized in a UV transilluminator (Mansour et al., 2017).

V- Antibiotic sensitivity Test:
The disk diffusion technique was used according to (Mohanty and Cock, 2010), to perform the antimicrobial susceptibility test for enterotoxigenic Staph. aureus strains, 0.1ml of bacterial suspension (1x10⁵ CFU/mL) equivalent to (0.5 McFarland) was plated on surface Mueller-Hinton agar. The plates was left for 2-5minutes for dry then antimicrobial disks were placed on surface of agar and incubated at 35°C±2 for 24-48hrs. Different antimicrobials were used like: PencillinG, amoxicillin, amikacin, erythromycin, clindamycin, tetracycline, vancomycin and sulfamthaxazole-trimethoprim. The diameter of inhibition zone was measured for each antimicrobial agents used and the interpretation was compared with the measurements of CLSI (2018) for each antimicrobial was used.

RESULTS

The current results illustrated in (Fig.1) showed that Staphylococcal isolates were recovered from 54.40% (68/125) of raw milk samples and 49.33% (37/75) of local pasteurized milk samples on manniot salt agar.
The biochemical profile revealed that the coagulase production was clear in 38 isolates out of 68 isolates of raw milk samples and 18 isolates out of 37 isolates of local pasteurized milk samples. Most of coagulase positive staphylococcal isolates of raw and local pasteurized milk declared a thermonuclease activity with percentages 78.9% (30/38) and in 72.2% (13/18), respectively. The positive coagulase and thermonuclease isolates were submitted to Voges-Proskauer test to discriminate Staph aureus, 18 out of 30 raw milk isolates and 8 out of 13 local pasteurized milk isolates were positive for Voges-Proskauer test (Table2).

PCR by using specific primer for (nuc gene), confirmed the presence of Staph aureus DNA in 15 out of 18 isolates of raw milk identified positive biochemically and in 7 out of 8 isolates from local pasteurized milk (Table 2&Fig.2).

The data postulated in Table (3) and Fig (3&4) revealed that enterotoxin genes were detected in 8 out of 15 Staph. aureus isolates (53.33%) from raw milk samples and 3 out of 7 Staph. aureus isolates (42.86%) from local pasteurized milk samples, sea and sed genes were detected in most Staph. aureus isolates, sec gene could be detected only in the raw milk isolates while seb and see genes were not detected in any Staph. aureus isolates from both raw and local pasteurized milk.

Enterotoxigenic Staph aureus (8 from raw milk and 3 from local pasteurized milk) were tested for their susceptibilities to eight antimicrobial agents. The data postulated in Table (4) demonstrated that a large section of enterotoxigenic Staph. aureus isolates showed resistance to penicillinG (72.73%) and tetracycline (72.73%). Intermediate resistance were showed against clindamycin (27.27%) and erythromycin (18.18%). All isolates showed a high sensitivity to vancomycin (100%) followed by amikacin (90.91%), sulfamethoxazole- trimethoprim (81.82%) and amoxicillin (81.82%).

Tables:

Table1: Primers sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence 5’</th>
<th>‘3</th>
<th>Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuc</td>
<td>5’GTTATCAATGTCGGGTG’3</td>
<td>5’ CGGCACCTTTTCTCTCGG’3</td>
<td>267</td>
<td>Brakstad et al. (1992)</td>
</tr>
<tr>
<td>sea</td>
<td>F- 5’ GTATGGTGTGTTAAGCTAGAC’3</td>
<td>R- 5’ CCCTACAGTACGAGTTAAGG’3</td>
<td>102</td>
<td>Mehrotra et al. (2000)</td>
</tr>
<tr>
<td>seb</td>
<td>F-5’GTATGGTGTGTTAAGCTAGAC’3</td>
<td>R-5’CCCTACAGTACGAGTTAAGG’3</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>sec</td>
<td>F-5’AGATGAAGTAGGTGATTATGATGATGG’3</td>
<td>R-5’CACACTTTTCTAGATCAACC’3</td>
<td>451</td>
<td></td>
</tr>
<tr>
<td>sed</td>
<td>5’CCAATTAGGAGAAAAATAAAA’3</td>
<td>5’ATTGGATTTTTTTTCGTTC’3</td>
<td>278</td>
<td></td>
</tr>
<tr>
<td>see</td>
<td>5’AGGTATTTTTTTCACAGGTCAATC’3</td>
<td>5’CTTTTTTTTTTCTCGTGATAC’3</td>
<td>209</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Specificity of PCR method for Staph. aureus DNA in raw and local pasteurized milk isolates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Biochemical identification</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Staph aureus isolates</td>
<td>No. of positive isolates</td>
</tr>
<tr>
<td>Raw milk</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3: Enterotoxigenic genes in Staph. aureus isolated from raw and local pasteurized milk samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total No. of Staph. aureus isolates</th>
<th>No. of Enterotoxigenic Staph. aureus isolates</th>
<th>Enterotoxin genotyping pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>15</td>
<td>1</td>
<td>Sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Sed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Sea+Sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Sea+Sec</td>
</tr>
<tr>
<td>Local pasteurized milk</td>
<td>7</td>
<td>2</td>
<td>Sea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Sea+Sec</td>
</tr>
</tbody>
</table>
Table 4: Sensitivity of enterotoxigenic *Staph. aureus* isolated from raw and local pasteurized milk to different antimicrobials.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>No. of Enterotoxigenic <em>Staph. aureus</em> isolates (no = 11)</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resist</th>
</tr>
</thead>
<tbody>
<tr>
<td>PenicillinG</td>
<td></td>
<td>3(27.27%)</td>
<td>0(0%)</td>
<td>8(72.73%)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td></td>
<td>9(81.82%)</td>
<td>0(0%)</td>
<td>2(18.18%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td>10(90.91%)</td>
<td>0(0%)</td>
<td>1(9.1%)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>11(100%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Sulfamethoxazole-Trimethoprim</td>
<td></td>
<td>9(81.82%)</td>
<td>1(9.1%)</td>
<td>1(9.1%)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td></td>
<td>7(63.64%)</td>
<td>3(27.27%)</td>
<td>1(9.1%)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>3(27.27%)</td>
<td>0(0%)</td>
<td>8(72.73%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>7(63.64%)</td>
<td>2(18.18%)</td>
<td>2(18.18%)</td>
</tr>
</tbody>
</table>

Figures:

Fig. (1): Incidence of *Staphylococcus* spp. in raw and local pasteurized milk samples on mannitol salt agar

Fig. (2): Amplified profile of *Staph. aureus* DNA positive for nuc gene at 267bp. M: Gel Pilot 100 bp ladder (QIAGEN, no. 239035), Lane1: positive control, Lane: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 positive isolates, Lane 14: negative control.
**DISCUSSION**

*Staph. aureus* is the principle cause of food poisoning and clinical infections in humans and animals (Chiang *et al.*, 2006). Enterotoxigenic *Staph. aureus* in milk possess a public health problem to consumers. So the identification of such strain should be used as a part of a risk in analysis of milk (Zouharova and Rysanek, 2008).

The conventional identification of *Staphylococcus* spp. based on many diagnostic methods such as growing on selective media containing 8-10% NaCl, production of coagulase, thermostable nucleases and acetoin production (Kateete *et al.*, 2010) also Quinn *et al.* (2002) reported that mannitol salt agar is one from the important media used specifically in food microbiology for identification of *Staphylococcus* spp. *Staphylococcus* isolates were recovered from 54.40% of raw milk samples on mannitol salt agar (Fig.1). Nearly similar incidences of *Staphylococcus* spp. were showed by Ghaleb *et al.* (2005) 57.5% and Uddin *et al.* (2011) 50%. A higher results were recorded by Daka *et al.* (2012) and Duguma (2018) who found that the incidences of *Staphylococci* were100%, and 76.2% in raw cow milk samples, respectively. On opposing with, Donkor *et al.* (2007) and Tessaema and Tasegaye (2017) who recorded that 14.6% and 28.2% of raw milk samples were contaminated by *Staphylococcus* spp.

On the other hand 49.33% (37/75) of local pasteurized milk samples were contaminated by *Staphylococcus* spp. (Fig.1). Lower percentage was conceded by El-Jakee *et al.* (2013) who detected *Staphylococci* species with percentage of 16% in the pasteurized milk samples, pasteurization always done at 60–65°C for 30 minutes and this can't kill *Staph.*
Coagulase and thermonuclease tests are the criteria used by many laboratories for the identification of *Staph. aureus* (Kaplan 2003), but there were other coagulase and thermonuclease positive *Staphylococci* like *Staph. hyicus* and *Staph. Intermedius* (Downes and Ito, 2001 and Viçosa et al., 2010), these strains also caused intramammary infections in dairy cows and reached to milk (Roberson et al., 1996).

Voges-Proskauer is an important test in determination of *Staph aureus* from other coagulase and thermonuclease positive isolates (Quinn et al., 2002; Arora, 2003 and Vos et al., 2009). These findings were supported our following results, the positivity of Voges-Proskauer were recorded in 18 isolates from raw milk samples and 8 isolates from local pasteurized milk samples (Table2). Higher results were illustrated by Rusenova and Rusenov (2017) who found that 73% of coagulase positive *Staphylococcal* strains isolated from different animals were Voges-Proskauer positive. This variation may back to level of contamination, number of samples and method of isolation (Fagundes and Oliveira, 2004).

Most of traditional methods for the identification of *Staph aureus* not reached to accurate identification for important veterinary pathogens. PCR assay can be used as a rapid and sensitive diagnostic method for diagnosis of *Staph aureus* in raw milk samples and it can be used in convincing accurate pasteurization methods of milk as a main food source (Brakstad et al.,1992). The PCR amplification of the *nuc* gene has a potential for the rapid and accurate diagnosis of *Staph aureus* infections (Kilic et al.,2010).

In our study, we used *nuc* gene primers in confirmation the presence of *Staph. aureus* DNA. This gene is a specific genetic marker for detection and confirmation of *Staph. aureus* (Hedge et al., 2013 and Hu et al., 2013). Furthermore, some previous studies suggested that there was a relationship between enterotoxin production and presence of *nuc* gene which considered as an indicator of food contamination with enterotoxigenic *Staph. aureus* (Tamarapu et al., 2001 and Cremonesi et al., 2005).

The data shown in table (2) and fig (2) cleared that PCR confirmed the presence of *staph aureus* DNA in 15 isolates and denied its existence in 3 isolates out of 18 isolates of raw milk also one isolate only of local pasteurized milk samples not confirmed as *staph aureus* by using specific primer (*nuc* gene), these results in accordance with Karahan and Cetinkaya (2007) in addition Bennett and Lancette (1998) reported that most international standards specially FDA found that PCR is the most essentially
equivalent method in detection of Staph aureus also Speers et al. (1998) found that the sensitivity of biochemical tests was low in compared to PCR, furthermore genetic method not necessarily correspond to the same results of conservative phenotypic tests (Bosshard et al., 2004).

Staph aureus microorganisms are able to produce enterotoxins which pose a risk factor on public health (Wu et al., 2016) and most of the food industry contaminated by Staph aureus containing SEs genes, especially moist foods containing starch and protein, such as meat, poultry products and milk (Tamarapu et al., 2001).

The results postulated in Table (3) and Fig. (3) showed that SEs genes were detected in 53.33% of Staph aureus isolates from raw milk samples. This result is in agreement with this reported by Jorgensen et al. (2005), Zouharova and Rysanek (2008) and Murphy et al. (2010). Lower recovery of enterotoxigenic Staph aureus was reported by EI-Jakee et al. (2013) and Mansour et al. (2017) 35.7% and 26.1% respectively. While Rahimi and Alian (2013) recorded a higher percentage of enterotoxigenic Staph aureus isolates (75%).

On the other hand, 42.86% of Staph aureus isolates from local pasteurized milk samples were enterotoxigenic isolates (Table3 & Fig. 4). Higher percentage was recorded by Breurec et al. (2010) 90% while EI-Jakee et al. (2013) found that no isolate showed a positive result for enterotoxin genes.

In our study, it was noticed that sea and sed genes were the most detectable genes in most Staph aureus isolates, sec gene could be detected only in two isolate of raw milk while seb or see genes were not detected in any isolate. These results supported Rall et al. (2008) and Carfora et al. (2015).

Pasteurized milk may also be disposed to toxin production because in various situations, the shop owners turn off the chillers at night to save electricity, leaving the product exposed to different degree of temperature as cited by Chapaval et al. (2010) also the highest risk of SEs production is associated with storage the pasteurized milk at room temperature, incubation (Janštova et al., 2012).

The classical Sea and Sed genes were a common concern in cases of Staphylococcus food poisoning (Tamarapu et al., 2001). Normanno et al. (2005) found that the sea gene is the most frequent SEs genes observed among enterotoxigenic strains of Staph aureus and the more common SEs in milk (Chiang et al., 2006), the presence of Staph aureus strains have sea gene isolated from milk may be due to the bad handling during milking and packaging the products, because sea gene is more common in human isolates than animal origin (El-Baradie, 1993).

In this study, the absence of Staph aureus isolates harbored seb or see genes were recorded in previous studies (Marija et al., 2016; Karahan et al., 2009; Neder et al., 2011; EI-Jakee et al., 2013 and Rahimi and Alian, 2013).

The development of Antibiotic resistance among the bacteria poses a problem of concern. Many original studies have exposed an increasing concern towards the existence of several antibiotic resistant of Staph aureus isolates in worldwide. So, our study was concerned by determining the susceptibilities of enterotoxigenic Staph aureus isolates against different families of antimicrobials. Table (4) demonstrated that a high resistance of these isolates were recorded against penicillin (72.73%) and tetracycline (72.73%) followed by clindamycin (27.27%) and erythromycin (18.18%), while all isolates were fully susceptible to vancomycin. Our results were consolidated by Gündgan et al. (2006) Pereira et al. (2009) and Waters et al. (2011), who noticed that multidrug resistance were shown against tetracycline, Penicillin and ampicillin. Abera et al. (2010) and Thaker et al. (2013) reported a high resistance to Penicillin-G 94.4% and 100% respectively, while Asimwne et al. (2017) reported a high resistance to tetracycline (73.2%).

The high resistance against penicillin was explained by Lee (2003) who returned the causes to the presence of resistance genes that coded for an alteration of penicillin-binding protein 2a which reduced the affinity for β-lactam antibiotics, another causes were recorded by Yamamoto et al. (2013) such as Staph aureus harbor a several antibiotic resistant plasmids that may lead to the phenotypes resistance.

Additionally, the mistreatment of infection by different antibiotics in dairy farms is known to be one of the major factors responsible for the multiple drug resistant of bacteria worldwide especially ampicillin and tetracycline were the mostly used on dairy cattle (Chee-Sanford et al., 2009).

CONCLUSION

Phenotypic methods not reach to a high grade in specificity of Staph aureus identification while, PCR play a confirmative role in detection of Staphylococcus aureus and focus a light on presence of enterotoxin types of these isolates in raw and local pasteurized milk which consider a public health problem. The existence of Staph aureus resistance to some antibiotic specially penicillin in milk reflect our need to management practices and appropriate sanitary procedures to be during milking operations also we suggest the occurrence of Staph aureus in local pasteurized milk may not only back to expire...
date but attributed to improper handling and storage temperature.

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الكشف عن جينات الأنتيروتوكسين في المكورالعنقودي الذهبي المعزول من البان الأبقار

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