Prevalence of Toxigenic and Methicillin Resistant Staphylococci in Poultry Chain Production

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ABSTRACT

Staphylococci are a worldwide cause of human and animal infection and are considered to be one of the most common causes of infections in birds. Enterotoxins produced by some staphylococcal species were recognized as a causative agent of staphylococcal food poisoning (SFP). Only enterotoxins produced by Staphylococcus aureus were as yet well characterized. Much less is known about enterotoxigenic potential of coagulase-negative species of genus Staphylococcus (CNS). It has been reported that enterotoxigenic CNS strains have been associated with human and animal infections and food poisoning. Samples collected from chicken production cycle (unhatched eggs, baby chicks, broilers, chicken meat and table eggs) in Luxor, Egypt were tested to investigate the presence of Staphylococcus species and detection of their enterotoxines genes with more special attention for detection of methicillin resistance gene (meca). Samples were tested for S. aureus and CNS on the basis of cultural and biochemical properties and confirmed by PCR amplification of 16S rRNA and clfa gene. Results showed that the presence of Staphylococci were 50/150 (33.3%), 14% of the samples were S. aureus (21/150), while, 19.33% were CNS (29/150). meca gene was detected in 66.7% and 51.7% among S. aureus and CNS respectively. Enterotoxines genes (seb, sec and see) were found in 5 (23.8%) of S. aureus and CNS with a percent of (9.5%) for seb and sec and (4.8%) for see, while sec and see were found in 6 (20.6%) of CNS. With a percent (10.3%) for each.

(30 samples were collected from each type (unhatched eggs, baby chicks, broilers, chicken meat and table eggs).

**Isolation of Staphylococci was done according to Sneath et al. (1986)**

The collected samples were inoculated in BPW (Difco), cultured onto Mannitol Salt agar (Difco) then incubated for 24-48 hours at 37°C. The resulted colonies were examined for identifying morphological characteristic appearance of Staphylococcus species.

**Identification and characterization of coagulase positive and negative Staphylococcus Species**

The isolates were identified according to MacFaddin (2000) by using conventional techniques such as: catalase test, oxidase test, growth at 10% NaCl, Mannitol fermentation, coagulase test as well as using PCR by detection of 16s rRNA gene specific for genus staphylococcius and clfA gene specific for S. aureus. (Mason et al., 2001).

**Serotyping of Staphylococcus isolates**

Coagulase negative staphylococci were selected and serotyped using INTEGRAL SYSTEM STAFILOCOCCHI kit based on biochemical tests (NCCLS, 2004).

**Detection of enterotoxins and mecA by Polymerase chain reaction (PCR)**

**Extraction**

All coagulase positive staphylococci CPS and CNS isolates were extracted according to QIAamp DNA mini kit (instructions (Qiagen, Germany)).

**Preparation of Master Mix**

According to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit, the primers used have specific sequence and amplify specific products as shown in Table 1.

**Cycling conditions of the primers during cPCR**

Temperature and time conditions of the primers during PCR are shown in Table 2.

**Results**

A total of 50 (33.3%) isolates were identified as Staphylo-

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**Table 1. Oligonucleotide primers sequences used to detect specific genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Length of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea</td>
<td>GSEAF-1</td>
<td>GOTTATCAAATGCGGGGTGG</td>
<td>102 bp</td>
<td>Mehrotra et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>GSEAR-2</td>
<td>CGGCACTTTTTTCTCTCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sec</td>
<td>GSECF-1</td>
<td>AGATGAAGTATTTTATGTTA</td>
<td>451 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSEC-2</td>
<td>CACACTTTTGAATCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sed</td>
<td>GSED-1</td>
<td>CCAATAAAGGAAATAAAG</td>
<td>278 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSED-2</td>
<td>ATTGGTAATTITTTCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>See</td>
<td>GSEE-1</td>
<td>AGGTTTTCACAGGTCATCC</td>
<td>209 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSEE-2</td>
<td>CTTTTCATCTGGGTAACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecA</td>
<td>MecA-F</td>
<td>GTA GAA AIG ACT GAA CTT CCG GTA A</td>
<td>310 bp</td>
<td>McClure et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>MecA-R</td>
<td>CCA ATT CAA CAT GTG TCT GGT TAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16S rRNA-F</td>
<td>CCTATTAGACTCAGGATAACCTCCGG</td>
<td>791 bp</td>
<td>Mason et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA-R</td>
<td>CTTGACTCCGTCACCTGGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>clfA</td>
<td>clfA-F</td>
<td>GCCAAAATCAGCGACACAAACGGAAAAG</td>
<td>638 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>clfA-R</td>
<td>CTGTATCTCCAGCCATATAATGGTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Temperature and time conditions of the primers during PCR.**

<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>mecA</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>50°C 30 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 7 min.</td>
</tr>
<tr>
<td>Enterotoxins genes</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>16S rRNA and clfA</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 45 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
</tbody>
</table>
coccus species, 14% of the samples were coagulase positive \textit{S. aureus} (21/150), while 19.33% were CNS (29/150). Coagulase positive \textit{S. aureus} revealed from 16.7% (5/30) of table eggs, 20% (6/30) of unhatched eggs, 13.3(4/30) of baby chicks and 10% (3/30) of broilers and chicken meat, while CNS revealed from from 10% (3/30) of table eggs, 26.7% (6/30) of unhatched eggs, 13.3% (4/30) of baby chicks, 33.3% (10/30) of broilers and 13.3% (4/30) of chicken meat as shown in Table 3 and Fig. 1.

The INTEGRAL SYSTEM STAFILOCOCCHI kit was used for identification of CNS isolates. The results were as follow, out of 29 CNS isolates, 10 isolates were \textit{S. xylosus} (34.49%), 5 \textit{S. warneri} (17.25%), 3 isolates of each of \textit{S. epidermidis}, \textit{S. saprophyticus}, \textit{S. simulans} and \textit{S. hominis} (10.34%) and 2 isolate of \textit{S. capitis} (6.9%).

\textit{mecA} gene was detected as 66.7% and 51.7% among \textit{S. aureus} and CNS respectively and it was found in CNS isolate as follow \textit{S. xylosus}, \textit{S. warneri}, \textit{S. epidermidis} and \textit{S. capitis} with the percentage 50%, 60%, 33.3%, 100% respectively and \textit{S. simulans} and \textit{S. hominis} with 66.7%.

Enterotoxins were found in 5 (23.8%) of \textit{S. aureus} as following: \textit{seb} and \textit{sec} (9.5%) and \textit{see} (4.8%) and found in 6 (20.6%) of CNS as following: \textit{sec} and \textit{see} (10.3%). \textit{sec} was detected in 2 isolates of \textit{S. xylosus} and 1 isolate of \textit{S. simulans}. \textit{sec} was detected in \textit{S. xylosus}, \textit{S. warneri}, \textit{S. simulans} and \textit{S. hominis} as shown in Table 4 and Figs 2, 3.

![Fig. 1. PCR result for the 16S rRNA gene (791bp) and clfa gene (638bp). Lane L: DNA ladder, Lane Neg: control –ve, Lane pos: control +ve (S. aureus strain), lane 3: non staph. Isolate, Lane 1,2,4,5,6,7,8,9 and10: CNS isolates, Lane 11,12,13,14 and 15 S. aureus.](image1)

![Fig. 2. PCR result of \textit{mecA} gene among staphylococcus isolates. Lane L: ladder, lane pos: control positive, lane neg: control negative lane 1,3,4,5,6,7,8,9,10,12,13,14 (+ve mecA), lane 2,11,15 (-ve mecA)](image2)

Table 3. Occurrence of \textit{S. aureus} and coagulase negative staphylococci from examined samples

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>No. of examined samples</th>
<th>Coagulase positive \textit{S. aureus}</th>
<th>CNS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Table eggs</td>
<td>30</td>
<td>5</td>
<td>16.7</td>
<td>3</td>
</tr>
<tr>
<td>Unhatched eggs</td>
<td>30</td>
<td>6</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Baby chicks</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
<td>4</td>
</tr>
<tr>
<td>Broilers</td>
<td>30</td>
<td>3</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Chickens meat</td>
<td>30</td>
<td>3</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>21</td>
<td>14</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 4. Occurrence of enterotoxin genes and \textit{mecA} gene among the staphylococcus isolates.

<table>
<thead>
<tr>
<th></th>
<th>No. of examined samples</th>
<th>\textit{seb}</th>
<th>No.</th>
<th>%</th>
<th>No.</th>
<th>%</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{CPS (S. aureus)}</td>
<td>21</td>
<td>2</td>
<td>9.5</td>
<td>2</td>
<td>9.5</td>
<td>1</td>
<td>4.8</td>
<td>14</td>
</tr>
<tr>
<td>\textit{S. xylosus}</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. warneri}</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. epidermidis}</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. saprophyticus}</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. simulans}</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>33.3</td>
<td>1</td>
<td>33.3</td>
<td>2</td>
</tr>
<tr>
<td>\textit{S. hominis}</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. capitis}</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>4</td>
<td>8</td>
<td>29</td>
</tr>
</tbody>
</table>

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and mucosa of humans and animals while some are free living. CNS (60%) was higher than that of CPS (40%). The high percentage of CNS was high (58%) compared to CPS (42%) and this agreed with Goja and Pranab (2016), the latter found that the percentage of CNS was high (53%) compared to Da cunha et al. 2003. This similar result agreed with Febler et al. (2012), who isolated S. aureus from newly hatched at a prevalence of 17.6% and 15% respectively and disagreed with Al-khalaf et al. (2011), who isolated S. epi-dermidis and S. xylosus were isolated from whites, yolks and shells of eggs.

In the present study, staphylococci were isolated from 30 unhatched eggs (18 and 21 day) dead and live embryo in 14(46.67%). S. aureus were 6 (20%) and coagulase negative staphylococcus isolates were 8(26.7%). (37.5% S. xylosus, 12.5% S. hominis, 12.5% S. simulans, 12.5% S. saprophyticus and 25% S. warneri) but (Babaca, 2014) isolated staphylococcus species from dead-in-shell chicken in 21.6%.

S. aureus contamination is very important cause of arthritis in chicks and early chick mortalities (Abd El-Latif, 1995). Examination of baby chicks revealed that 8 (26.6%) staphylococcus species were isolated from 30 baby chicks (1-7days). 4 (13.3%) from each S. aureus and CNS. Coagulase negative staphylococcus isolates were 2 (50%) S. xylosus. 1 (25%) S. saprophyticus and 1 (25%) S. epidermidis, and this result agreed with Abd El-Gali et al. (1984) and Azmy (1996) who isolated S. aureus from newly hatched at a prevalence of 14.7% and 15% respectively and disagreed with Al-khalaf et al. (2011), who isolated S. epi-dermidis and S. xylosus were isolated from hatchings of 150 and Shareef et al. (2009) isolated S. aureus with a percentage of 29.1% from one day old chick samples.

Staphylococcal osteomyelitis has been recognized as one of the major problems in broiler chickens as reported by Skeeles (1997). In these study staphylococcus species were detected in 13 out of 30 (43.3%) from broiler, 3 (10%) were S. aureus and 10 (33.3%) strains were CNS (S. xylosus) (5), S. simulans (2), S. capitis (2), S. warneri (1), and this agreed with Sobhy et al. (2014), who detected S. capitis, S. simulans, S. sciuri, S. haemolyticus, S. xylosus and S. saprophyticus from broilers chicken and agreed with Youssef and Hamed (2012), who isolated S. aureus (11.7%) from apparently healthy broilers in Ismailia governorate. On the other hand, they were consist-ent with the results of Rasheed (2011) that isolated S.aureus at the percentage of 50.98% from different broiler chickens farm, this may be due to the samples obtained from healthy and diseased bird.

The result of this study revealed that staphylococcus species found in 7 (23.3%) from 30 raw chicken samples (breast, neck and thigh) 2 (6.7%) strains were S. aureus and 4 (13.3%) were CNS (S. hominis, S. simulans, S. warneri) and this result agreed with Mohammad et al. (2014) that isolated S. au-reus from15.7% raw chicken meat. also agreed with Sumru and Tugba (2011), who found CNS in 25.2% chicken. and disagreed with Yurdakul et al. (2013) who isolated 22 coagulase negative staphylococci from 50 chicken meat samples and this may be due to difference of the sample collection site.

It was observed that 66.7% (14/21) and 51.7% (15/29) of S. aureus and coagulase negative staphylococcus isolates (CNS) were positive for detection of meca gene. The results were agreed with Febler et al. (2012), who isolated MRSA (Methicillin-resistant Staphylococcus aureus) from (50.0%) of staphylococcus isolates, Helen et al. (2011) detected MR-CNS in 48.3% of examined samples and Koksai et al. (2009), who observed that 67.5% of CNS isolates were Methicillin-resistant, while the result disagreed with Akbar and Anai (2013), who detected meca gene in 18.18%, and EL-Shareek and Ali (2012) that found the gene in 29.6%.

Staphylococcal enterotoxins (SE) constitute a family of biologically and structurally related toxins and the ingestion of these toxins results in gastrointestinal effects such as nausea, vomiting, diarrhea and abdominal pain. The SEs are the main cause of many outbreaks of food borne diseases (Lamaita et al., 2001).

Although enterotoxins are produced mainly by coagulase positive staphylococci, some coagulase-negative staphylo-cocci (CNS), involved in a variety of human and animal infections (Kloos et al., 1995), CNS can contaminate foods because...
humans are common carriers of these microorganisms and some may be related to specific human infections (Bergdoll, 1995).

The data illustrated that 5/21 S. aureus isolates (23.8%) and 6/29 CNS strains (20.7%) showed positive results for presence of enterotoxin genes. Three classical enterotoxin genes (seb, sec and see) were detected in the present work. This was agreed with Kitai et al. (2005) (21.7%); Naffa et al. (2006) (23%) ; Holmberg and Blake (1984), (26.5%) of S. aureus isolates produced SE and Çepoglu et al. (2010), who found that 20% of 20 CPS was produced staphylococcal enterotoxin.

In this study seb enterotoxin was produced by 9.5% of S. aureus and was not produced by CNS. The result agreed with Polledo et al. (1985), who found that the distribution of enterotoxins seb were 8 (9.6%) of CPS strains and disagreed with Kitai et al. (2005), who detected seb in 64.1% of S. aureus and Rasoul et al. (2015) in 4.1%. Staphylococcal enterotoxin B (SEB) is the toxin most commonly associated with classic food poisoning. CDC (2014) reported that SEB has been studied as a potential biological warfare agent because it can easily be aerosolized; it is very stable; and can cause widespread system damage, multi-organ system failure, and even shock and death when inhaled at very high dosages. However, SEB is classified as an incapacitating agent because in most cases aerosol exposure does not result in death but in a temporary, though profoundly incapacitating, illness lasting as long as 2 weeks (Ulrich et al., 1997).


Studies have shown that sec is the most thermotable enterotoxins, followed by seb and sea (Notermans et al., 1988), while sec enterotoxin was found in this study by 9.5% and 10.3% of S. aureus and CNS respectively. This agreed with Polledo et al. (1985), who found sec in 6.4% from CPS strains and Kitai et al. (2005), who found sec in 10.3% of S. aureus and disagree with Enas et al. (2016) that detected sec gene in 23% of S. aureus isolates.

Staphylococcal food poisoning outbreak where see has been confirmed as the causative agent (Ostyn et al., 2010). In this study see enterotoxin was detected in 4.8% and 10.3% from S. aureus and CNS respectively. This was agreed with Holmberg and Blake (1984), who detected see in 4.3% of S.aureus. However, Asadollahi et al. (2014) who reported a high level of see gene (31%). Other studies reported low level of see gene distribution (2.4%) (Polledo et al., 1985).

The genes seb and sec were found in similar percentages (Rall et al., 2010b), which agreed with results from this study, which also demonstrated the presence of see and sec genes in 30% of S. xylosus, which agreed with Da cunha et al. (2006), who detected sec gene in one S. xylosus isolate. S. xylosus, are used as a starter culture in fermented meat products (Montel et al., 2000).

In present study S. warneri carried see enterotoxin gene in 20%. S. simulane produced sec and see enterotoxin in 33.3%. However, S. epidermidis, S. saprophyticus, S. hominis and S. capitis not produced enterotoxins.

Valle et al. (1991) found a toxigenic capacity in 45 (16.5%) CNS isolates, including S. epidermidis, S. haemolyticus, S. warneri, and S. xylosus. Stepien et al. (2016) suggested a strong association between coagulase-negative S. simulans and endocarditis in broiler chicken.

Conclusion

From above mentioned data, it was observed that coagulase positive Staphylococci and coagulase negative staphylococci contain MecA gene and different types of enterotoxin genes. So, attention should be given to CNS because there is no more studies on it.

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