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# Detection of Enterotoxin A gene in *Staphylococcus aureus* isolated from Broiler farms in Bohera Governorate

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**ABSTRACT:**

*Staphylococcus* causes disease conditions in poultry and widely distributed. Broilers may suffer from *Staphylococcus aureus* infection and its enterotoxins resulting in a major public health hazard and economic losses. This study aimed to detect the presence of *Staphylococcus aureus* producing Enterotoxin A in some broiler farms. Samples were collected from 200 broiler chickens, 60 apparently healthy (nasal swabs, tracheal swabs & cloacal swabs), and 140 diseased broilers (lung, liver, heart blood, spleen & thigh bone marrow). Samples were collected aseptically from 10 randomly selected broiler farms in Bohera Governorate, Egypt, and were subjected for bacteriological examination for isolation and identification of *S. aureus*. The results of isolation of *Staphylococcus* from broiler chicken samples revealed that out of 200 bacteriologically examined broilers obtained from a total of 98 (49%) *Staphylococcus* isolates were isolated, 23 (38.3%) from apparently healthy and 75 (53.6%) from diseased birds. Also, out of the 98 *Staphylococcus* isolates 20 (20.4%) were identified as coagulase positive *S. aureus* of which 4 isolates (17.4%) isolated from apparently healthy birds and 16 isolates (21.3%) from diseased ones. In apparently healthy birds, *S. aureus* was isolated from nasal swab, tracheal swab and cloacal swab samples with percentages of 10%, 12.5% and 40%, respectively. In diseased birds, S. aureus was isolated from lung, liver, heart blood, spleen and thigh bone marrow samples with percentages of 15%, 24%, 20%, 20% and 26.7%, respectively. Antimicrobial resistance against 10 antimicrobial agents was performed and revealed that ciprofloxacin was the most effective antibiotic with susceptibility percentage of 95% followed by levofloxacin with sensitivity percent 75% and enrofloxacin, gentamycin (70% for each). All the *S. aureus* isolates were completely resistance against

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trimethoprim-sulfamethoxazole (100% resistance), followed by streptomycin, tetracycline and erythromycin with resistant percentages 80% for each. Variable degrees of sensitivity were reported for colistin sulfate and penicillin with percentages of 55 % and 45%, respectively. Molecular identification of *S. aureus* Enterotoxin A (SEA) gene by PCR using specific primers revealed that 3 isolates out of 10 (30%) were positive for Enterotoxin A (SEA) gene. Our study concluded that broilers may act as a source of *S. aureus* which causes diseased problems leading to large economic losses, and broilers may be a source of (SEA) gene which can transmit to human causing illness.

## INTRODUCTION:

Most *Staphylococcus* species are pathogenic and capable of producing toxins that result in health problems for both humans and animals. *Staphylococcus aureus* is naturally found as normal flora on animals and humans' skin **(Fetsch and Johler. 2018)** and on mucosal surfaces such as the upper respiratory tract, alimentary tract, and genitourinary tract. It easily spread between different animal species, and between humans and animal species **(Weese. 2010).** S. aureus is an important foodborne pathogen associated to food intoxication and other multiple infections **(Rashid et al. 2021).** Staphylococcus infection cause both systemic infection or localized lesions. It causes arthritis, synovitis, chondronecrosis with osteomyelitis when invade the metaphyseal area of joints. Also, it causes omphalitis, gangrenous dermatitis, localized abscesses, and endocarditis **(Abou-Zahr et al. 2018).**

*S. aureus* is a gram-positive, catalase-positive, coccoid bacteria that appears in grape -like clusters on stained smears **(Todar. 2008**). S. aureus produces some virulent toxins and enzymes and has some virulent surface proteins, which together contribute to its pathogenicity since it is one of the most significant and widespread foodborne causes and is implicated usually of food poisoning .

Antibiotics are dangerously overused in the veterinary industry, which results in the emergence of human-transmittable, multidrug-resistant *S. aureus* strains **(Wafaa. 2021)**. Resistance to frequently used antibiotics was reported in bacteria present in broiler since the introduction of these antimicrobial agents **(Mamza et al. 2010),** including penicillin, erythromycin, and tetracyclines, are widely used for treating *Staphylococcal* infections in broiler **(White et al. 2003)**. The proportion of resistant bacteria are increasing in the poultry due to excessive use of antibiotics in the broiler farms **(Lukášová and Šustáčková. 2003).** Enterotoxin-producing *S. aureus* is the most common cause of food-borne human illness throughout the world **(Do Carmo et al. 2004).**

*S. aureus* enterotoxin A (SEA) in broiler chickens is a potential health risk for consumers **(Olimpia et al. 2006).** SEA is capable of causing gastroenteritis in humans, thereby making them the causative agents of Staphylococcus food poisoning (SFP) **(Wu and Su. 2014)**. In addition, SEA is resistant to proteolytic enzymes and normal heat processing; even if *S. aureus* have been sterilized, the biological activity of SEA remains unchanged. According to regulation **(EC No. 1441/2007**), *S. aureus* strains that produces enterotoxin A (SEA), are the most common type of *S. aureus* enterotoxins in food products **(Zeaki et al. 2014).**

## MATERIALS and METHODS:

### Collection of samples:

Samples were collected aseptically from a total of 200 broiler chickens (60 apparently healthy and 140 diseased) from 10 randomly selected broiler farms in Bohera Governorate, Egypt. The diseased broiler chickens appeared weak with reduction of body weight, dehydration and swollen joints. The collected samples included nasal swabs, tracheal swabs & cloacal swabs (from apparently healthy broilers), lung, liver, heart blood, spleen & thigh bone marrow (from diseased broilers) as shown in table (1), then kept in ice box then immediately transported to the laboratory.

Table 1. samples collected for staphylococcus isolation from broiler chickens :

| Broiler cases | Samples | |
|---|---|---|
| | No. | % |
| Apparently healthy | 60 | 30 |
| Diseased | 140 | 70 |
| Total | 200 | 100 |

No.= number of broiler cases. %= percent of broiler cases.

***S. aureus* Isolation and Identification:**

For isolation of *Staphylococci* the collected samples were inoculated on nutrient broth and incubated at 37°C for 24 hours and subcultured on blood agar, mannitol salt agar and Baird Parker agar media according to **Quinn et al. (2002)**. Identification was carried out according to their colony characteristics, microscopic appearance with Gram stained, and biochemical characterization including positive catalase reaction, hemolysin production, and coagulase test with rabbit plasma (tube method) for initial differentiation of *S. aureus* and coagulase negative *Staphylococcus* isolates according to **Quinn et al. (2002)**.

**Determination of antimicrobial susceptibility:**

The antimicrobial susceptibility of *S. aureus* isolates were determined by standard disc diffusion method according to the recommendations of Clinical and Laboratory Standards Institute ***(*CLSI. 2022)**. For this purpose Ten Antimicrobial agents were used: penicillin (5 ug), ciprofloxacin (5 ug), colistin sulfate (25 ug), gentamicin (30 ug), streptomycin (10 ug), sulfamethoxazole-trimethoprim (23.73–1.25 ug), tetracycline (30 ug), levofloxacin (5 ug), enrofloxacine (5 ug) and erythromycin (15 ug). **(Oxoid, Hampshire, UK).** The results were interpreted according to **NCCLS. (2002).**

**Molecular characterization of *S. aureus* enterotoxin A isolates:**

**DNA extraction:**

DNA extraction from samples was performed using the QIAamp DNA Mini kit **(Qiagen, Germany, GmbH**) with modifications from the manufacturer's recommendations according to **Eid and Erfan. (2013)**. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}$C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's *recommendations according to* ***Eid and Erfan. (2013)***. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

**Oligonucleotide Primers:**

Primers used were supplied from **Metabion (Germany)** are listed in table (2).

**PCR amplification:**

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

**Analysis of PCR Products:**

The products of PCR were separated by electrophoresis on 1.5% agarose gel (**Applichem, Germany, GmbH**) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (**Fermentas, Germany**) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 2. Primers sequences, amplicon sizes and cycling conditions of *S. aureus* enterotoxin A (SEA) gene *(*Mehrotra et al. 2000):

| Primers sequences | Amplified segment (bp) | Primer Den. | Amplification (35 cycles) Sec. den. | Ann. | Ext. | Final extension |
|---|---|---|---|---|---|---|
| GGTTATCAATGTGCGGGTGG<br>CGGCACTTTTTTCTCTTCGG | 102 | 94˚C<br>5 min | 94˚C<br>30 sec. | 57˚C<br>30 sec. | 72˚C<br>30 sec. | 72˚C<br>7 min. |

Den., denaturation, Sec. den., secondary denaturation, Ann., annealing & Ext., extension.

**RESULTS:**

The results of isolation of *Staphylococcus* from broiler chicken samples revealed that out of 200 bacteriologically examined broilers obtained from a total of 98 (49%) *Staphylococcus* isolates were isolated, 23 (38.3%) from apparently healthy and 75 (53.6%) from diseased birds. Also**,** out of the 98 *Staphylococcus* isolates 20 (20.4%) were identified as coagulase positive *S. aureus* of which 4 isolates (17.4%) isolated from apparently healthy birds and 16 isolates (21.3%) from diseased ones. On the other hand, 78 isolates (79.6%) of coagulase negative *Staphylococci* were isolated from both apparently healthy (19) and diseased birds (59) with percentages of (82.6%) and (78.7%) as shown in table (3).

Table 3. Prevalence of coagulase positive *S. aureus* in apparently healthy and diseased broiler cases:

| Broiler cases | Broiler cases number | Total number of *Staphylococci* | | Coagulase positive *S. aureus* | | Coagulase negative *Staphylococci* | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Apparently healthy | 60 | 23 | 38.3 | 4 | 17.4 | 19 | 82.6 |
| Diseased | 140 | 75 | 53.6 | 16 | 21.3 | 59 | 78.7 |
| Total | 200 | 98 | 49 | 20 | 20.4 | 78 | 79.6 |

No.= number of isolates. %= percentage .

Out of 23/60 (38.3%) cases *Staphylococci* found in apparently healthy birds, coagulase positive *S. aureus* isolated from 4/ 23 (17.4%) cases and coagulase negative *Staphylococci* isolated from 19/23 (82.6%) cases. The *S. aureus* was isolated from nasal swab, tracheal swab and cloacal swab samples with a percent of 1/10 (10%), 1/8 (12.5%) and 2/5 (40%), respectively, as shown in table (4).

Table 4. Prevalence of coagulase positive *S. aureus* in different samples of 60 apparently healthy broiler chickens.

| Source of collected sample | Total number of *Staphylococci* | | Coagulase positive *S. aureus* | | Coagulase negative *Staphylococci* | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| Nasal swabs | 10 | 16.7 | 1 | 10 | 9 | 90 |
| Tracheal swabs | 8 | 13.3 | 1 | 12.5 | 7 | 87.5 |
| Cloacal swabs | 5 | 8.3 | 2 | 40 | 3 | 60 |
| Total | 23 | 38.3 | 4 | 17.4 | 19 | 82.6 |

No.= number of isolates. %= percentage

Out of 75/140 (53.6%) cases *Staphylococci* found in diseased birds, coagulase positive *S. aureus* isolated from 16/75 (21.3%) cases and coagulase negative *Staphylococci* isolated from 59/75 (78.7%) cases. The *S. aureus* was isolated from lung, liver, heart blood, spleen and thigh bone marrow samples with a percent of 3/20 (15%), 6/25 (24%), 2/10 (20%), 1/5 (20%) and 4/15 (26.7%), respectively, as shown in table (5).

Table 5. Prevalence of coagulase positive *S. aureus* in different organs of 140 diseased broiler chickens:

| Source of collected sample | Total number of *Staphylococci* | | Coagulase positive *S. aureus* | | Coagulase negative *Staphylococci* | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| Lung | 20 | 14.3 | 3 | 15 | 17 | 85 |
| Liver | 25 | 17.9 | 6 | 24 | 19 | 76 |
| Heart blood | 10 | 7.1 | 2 | 20 | 8 | 80 |
| Spleen | 5 | 3.6 | 1 | 20 | 4 | 80 |
| Thigh bone marrow | 15 | 10.7 | 4 | 26.7 | 11 | 73.3 |
| Total | 75 | 53.6 | 16 | 21.3 | 59 | 78.7 |

No.= number of isolates. %= percentage

All *S. aureus* isolates (20) were tested for susceptibility to 10 antimicrobial agents. The results of antimicrobial sensitivity tests indicated that ciprofloxacin was the most effective antibiotic in our study with susceptibility percentage of 95% followed by levofloxacin with sensitivity percent 75% and enrofloxacin, gentamycin (70% for each). Conversely, all the *S. aureus* isolates were completely resistance against trimethoprim-sulfamethoxazole (100% resistance), followed by streptomycin, tetracycline and erythromycin with resistant percentages 80% for each. Variable degrees of sensitivity were reported for colistin sulfate and penicillin with percentages of 55 % and 45%, respectively, as illustrated in (Table 6).

Table 6. Antimicrobial susceptibility test of *S. aureus* isolates (No. = 20):

| Antimicrobial agents | Conc. of disc | Sensitive | | Resistant | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| Penicillin (P) | 5 ug | 9 | 45 | 11 | 55 |
| Ciprofloxacin (CIP) | 5 ug | 19 | 95 | 1 | 5 |
| Colistin sulfate (CT) | 30 ug | 11 | 55 | 9 | 45 |
| Gentamicin (GEN) | 10 ug | 14 | 70 | 6 | 30 |
| Streptomycin (S) | 10 ug | 4 | 20 | 16 | 80 |
| Sulphamethoxazole-trimethoprim (SXT) | 23.73–1.25 ug | - | - | 20 | 100 |
| Tetracycline (TET) | 30 ug | 4 | 20 | 16 | 80 |
| levofloxacin (LEV) | 5 ug | 15 | 75 | 5 | 25 |
| Enrofloxacin (ENR ) | 5 ug | 14 | 70 | 6 | 30 |
| Erythromycin (E) | 15 ug | 4 | 20 | 16 | 80 |

%, calculated according to the No. of tested isolates (20)

Detection of enterotoxin A gene for 10 isolates of *S. aureus* (SEA), revealed presence of 3 positive isolates, as showed in figure (1).

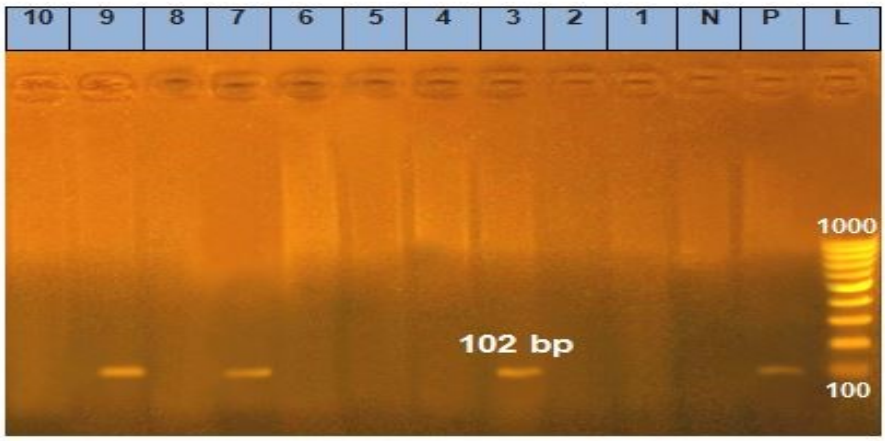


**Figure (1):** Agarose gel electrophoresis patterns showing PCR amplification products for the *S. aureus* enterotoxin A (SEA) gene. Lanes L, DNA molecular size marker (100-1000bp ladder; Bethesda Research Laboratories Inc., Gaithersburg, Md.), lanes 1, 2, 4, 5, 6, 8 & 10 (Negative). lanes 3, 7 & 9 (Positive).

## DISCUSSION:

*Staphylococcus* species are significant bacteria in the etiology of avian diseases **(Saleh et al. 2003).** Broiler chickens are regarded as an import-ant reservoir for pathogenic *S. aureus* strains. In the current study, 98 *Staphylococcal* isolates were isolated from 200 broilers with a percentage of (49%) which higher than that reported by **Masdooq et al. 2008** (15.2%), who isolated *Staphylococci* from pathogenic bacteria associated with respiratory diseases in poultry and nearly similar to the percentage detected by **(Abd El Tawwab et al. 2014)** who recorded 51.6% *Staphylococcal* species in broiler chickens.

In the current study, coagulase positive *S. aureus* were isolated in rate of 20.4% of both apparently healthy and diseased broiler chickens, these results were nearly similar to results of **(Abd El Tawwab et al. 2014** and **Elmossalamy et al. 2020)** which confirmed isolated coagulase positive *S. aureus* from 22.6% and 20% of the broiler chickens. Higher rates were recorded by **(Abd El-Tawab et al. 2017; Ali et al. 2017** and **Amen et al. 2019)** who recovered S. aureus in 66%, 90% and 74.07% of the tested broiler chickens, respectively.

Coagulase positive *S. aureus* was recovered from 4 samples (17.4%) of apparently healthy broiler chickens and 16 samples (21.3%) from diseased broiler chickens, these results were nearly in accordance with **(Abd El Tawwab et al. 2014)** who found coagulase positive *S. aureus* with a percent of 20.7% in apparently healthy broilers and 18.9% in diseased broiler chickens.

*S. aureus* in apparently healthy broiler chickens was mostly recovered from cloacal swab samples with an incidence of 40% followed by tracheal swab and nasal swab samples with prevalence of 12.5% and 10%, respectively, which agreed with **(Abd El Tawwab et al. 2014)**.

*S. aureus* in diseased broiler chickens was highly recovered from thigh bone marrow samples with an incidence of 26.7% followed by liver, heart blood, spleen and lung samples with prevalence of 24%, 20%, 20% and 15%, respectively, which agreed with **(Abd El Tawwab et al. 2014)**.

Widespread occurrence and persistence of antimicrobial-resistant *S. aureus* in livestock animals is a major public health concern, in humans and animals **(Becker et al. 2017)**. The marked and continuous use of the antimicrobi-

al agents during short period all over the raising period of broiler chickens not only to control and prevent disease but also for growth promotion and improved feed conversion efficiency **(Bertolatti et al. 2003)**. Antibiotic Sensitivity tests using 10 antibiotic agents revealed that ciprofloxacin was the most sensitive antibiotic followed by levofloxacin, enrofloxacin and gentamicin by percentage of 95%, 75%, 70% and 70%, respectively while the highest resistance was to the sulphamethoxazole-trimethoprim, tetracycline, erythromycin and streptomycin by percentage of 100%, 80%, 80% and 80%, respectively . These results were disagreed to that of **(Abd El Tawwab et al. 2014)** who found that the highest sensitivity rates were to vancomycin, amoxicillin + clavulinic acid and cephalothin by 84.5%, 83.8% and 78.4, respectively and the highest resistance were to ampicillin, oxacillin and penicillin by 75.7%, 73% and 70.2%, respectively. Our study revealed that 100% of *S. aureus* isolates were resistant to sulphamethoxazole-trimethoprim which agreed with **(Bakheet et al. 2018)** who tested *S. aureus* isolates against selected antibiotics commonly used in poultry farms and found that 100% of isolates were resistant to sulphamethazole -trimethoprim. They also recorded 100% of isolates were resistant to streptomycin which nearly similar to our results where we recorded 80% of *S. aureus* isolates resistant to streptomycin. **Bakheet et al. (2018)** also revealed that 100% of *S. aureus* isolates were sensitive to enrofloxacin which nearly similar to this study where 70% of *S. aureus* isolates were sensitive to enrofloxacin. **Amen et al. (2019)** revealed that *S. aureus* isolates were highly sensitive to ciprofloxacine, enrofloxacine and resistant to erythromycin and streptomycine which nearly similar to our results. The variances in resistance patterns are commonly due to factors which include differences in geographical locations, particular bacteria species involved, the animal production systems, the extent to which antibiotics are used, sampling techniques and period of sampling as reported by **(Adzitey et al. 2015).**

The enterotoxin genes are accessory genetic elements in *S. aureus*, meaning that not all strains of this organism are enterotoxin producing, they are encoded by mobile genetic elements including phages, plasmid and pathogenicity islands **(Martin et al. 2004**). SEA is the most common toxin involved in *Staphylococcal* food poisoning outbreak, although *Staphylococcal* endotoxins are generally considered to be heat stable **(Halpin-Dohnalek and Marth. 1989)**. From 10 isolates of *S. aureus* were detected by PCR for the SEA gene, there were 3 isolates positive for SEA gene originated from broiler chickens, that means that the broiler chickens may act as a source of SEA which agreed with **( Hassan-Aisha. 2007)** and **(Gad. 2004)** who stated that *S. aureus* isolates from broiler chickens produced SEA. Also **(Azevedo et al. 2009)** recorded that 30% of the examined *S. aureus* strains isolated from broiler were enterotoxigenic and produced SEA. **Elmossalamy et al. (2020)** revealed *S. aureus* strains isolated from broiler chickens have the ability to produce SEA which represent a public health hazard, and that agreed with our study.

## CONCLUSSION

*Staphylococcus* is one of the organisms that are widely distributed in broiler chicken farms. In this study, *S. aureus* was isolated with a percent of 20.4% from boiler chickens. Antimicrobial susceptibility test of the *S. aureus* isolates showed a multi-drug resistance, so it is important to continually monitor antibiotic susceptibilities. Also, further studies should be made to determine antimicrobial susceptibility test which should be carried regularly to determine the development of resistance against the commonly applied antibiotics used in field at broiler farm helping the clinician's choice of antibiotic to control infection.

Our study revealed the presence of SEA gene in broiler chickens, so broiler chickens should be considered as a reservoir of SEA and should be careful during dealing with broiler chickens which suspected to infection with SEA which may be transmitted to humans.

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