Identification and Molecular Characterization of *Staphylococcus Aureus* From Newly Hatched Imported Poultry

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Abstract
Bacteriological examination of 166 different flocks collected from 62, 52 and 52 imported duck, chick and poult flocks respectively, revealed that 15, 8 and 14 flocks were positive for *S.aureus* isolation with percentage of 24.2%, 15.4% and 26.9% respectively. In-vitro antimicrobial susceptibility testing for isolates was studied using disc diffusion method. All *Staphylococcus aureus* isolates were subjected to molecular detection using PCR to confirm the results of isolation. This data focusing on newly hatched imported poultry represent a risk of introducing *S. aureus* to the country. Effective control measures are required to mitigate the economic impact on the poultry industry and to prevent possible public hazards.

Keywords: *Staphylococcus aureus*; Antimicrobial susceptibility; imported poultry

Introduction

Staphylococci represented one of the most important bacterial pathogen where it is normal inhabitant of the skin and mucosal surface of the most important organs of mammals and birds ***(El-Jakee et al., 2008).*** In poultry Staphylococci caused severe economic losses in different forms, for example decreased body weight, decreased egg production and suffering from septicemia and osteomyelitis which lead to lameness, and condemnation of carcasses at slaughter ***(McNamee and Smyth 2000 and Andreasen, 2008).*** Moreover, food poising in human beings caused by *S. aureus* which considered as a major disease problem in poultry. Its enterotoxins are the main cause of food poisoning in human due to contamination of poultry carcasses at processing with *S. aureus* ***(Evans et al., 1983).***

antimicrobial drug resistance which is increased worldwide specially in *S. aureus* which appeared in many types of antimicrobial drug ***(Talbot***

et al., 2006 and Okonko et al., 2009). and the effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections ***(Nawaz et al., 2009).***
However, the standard conventional methods for isolation and characterization of microorganisms are still considered as methods for traditional confirmation of *S. aureus* and the accurate result is obtained by combination of conventional culture method followed by PCR ***(Velasco et al., 2014).*** The use of PCR in routine testing is reduces the time required to attain results ***(Brown, 2001).*** The specific gene encoding a surface associated fibrinogen binding protein is called *clfA* gene ***(McDevitt et al., 1994).***
Therefore the present work was planned to identify and characterize *Staphylococcus aureus* collected from apparently healthy newly hatched imported chicks, duckling and poults. Examine the susceptibility of isolates to broad range of antimicrobial agents and isolates confirmed by polymerase chain reaction technique.

Materials and methods

Samples

Samples were collected from 62 imported duck flocks, 52 imported chick flocks and 52 imported poults flocks, per each flock examined 15 bird pooled in 2 different samples (internal organs "liver, heart and lung", and yolk). The examined birds were submitted to the reference laboratory for veterinary quality control on poultry production. All samples used were collected under aseptic conditions and safety precautions to prevent cross contamination according to **(*Middleton et al., 2005).*** As in Table (1).

Bacteriological examination

Isolation and Identification of *Staphylococcus aureus* was done according to standard methods ***BAM: 2001*** *and* ***ISO 6888-1: (2003)***. Isolated colonies were identified morphologically, microscopically and biochemically according to. ***Sneath et al. (1986)*** *and* ***Quinn et al. (2002)*** Colony diameter <9 mm, Colony pigment (carotenoid) with Aerobic growth, Slide catalase test (+ve), Oxidase test (-ve), Mannitol fermentation (+ve), Tellurite reduction with lipase activity (+ve), Haemolysis (+ve) and most strains of *S.aureus* were β-haemolytic, tube Coagulase test (+ve) and Acetoin production (VP) (+ve).

Antibiotic sensitivity test:

The antibiogram of *S. aureus* isolates were done by disc-diffusion test. *S. aureus* tested against 14 antibiotics (Oxoid) and the interpretation according to **(CLSI/NCCLS, 2009**). As shown in Table (2).

Conventional PCR technique:

Extraction:
DNA of refreshed isolates was extracted using commercially available kit, **QIAamp DNA Mini Kit**, Catalogue no.51304.

Amplification.

16S rRNA and clf gene were amplified according to refernce mentioned in Table (3). For confirmation of the isolation.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis and loaded in each gel slot. A 100+ bp DNA Ladder **(Qiagen, Germany, GmbH)** were 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 (1) *Sources and numbers of examined samples*

Source of samples	Type of samples	No.of flock	No.of samples
Duckling flocks (15 duck/ flock)	Organs	62	1860
	Yolk		
Chick flocks (15 chick/ flock)	Organs	52	1560
	Yolk		
Poult flocks (15 poult/ flock)	Organs	52	1560
	Yolk		
Total	**166**		**4980**

Table (2) *Sensitivity test interpretation of S. aureus (CLSI/NCCLS, 2009).*

Antimicrobial Discs	code	Disc Potency Mg/disc	Interpretation		
			Zone diameter (mm)		
			Sensitive ≥	Intermediate	Resistant ≤
Amikacin	**Ak^{30}**	30 µg	17	15-16	14
Amoxicillin + Clavulinic acid	**Am+CL**	20-10µg	20	——	19
Ofloxacin	**Of^{5}**	5 µg	18	15-17	14
Clindamycin	**DA**	2 µg	21	15-20	14
Oxacillin	**O^{1}**	1 µg	13	11-12	10
Ceftriaxone	**CRO**	30 µg	21	14-20	13
Ciprofloxacin.	**CF^{5}**	5 µg	21	16-20	15
Doxycycline.	**DO^{30}**	30µg	16	13-15	12
Erythromycin	**E^{15}**	15 µg	23	14-22	13
Gentamicin.	**G^{10}**	10 µg	15	13-14	12
Norfloxacin.	**NX^{10}**	10 µg	17	13-16	12
Penicillin	**P^{10}**	10 I.U.	29	——	28
Tetracycline.	**T^{30}**	30	19	15-18	14
Trimethoprim-sulfamethoxazole	**SXT**	1.25-23.75µg	16	11-15	10

Table (3): *Annealing temperature of primers and the size of amplified products required for detecting the tested genes.*

Target gene	Primer sequence (5'-3')	Amplicon (bp)	References
Staphylococcal 16S rRNA	**F**:CCTATAAGACTGGGATAACTTCGGG **R**:CTTTGAGTTTCAACCTTGCGGTCG	**791**	**Mason *et al.*, 2001**
S. aureus clfA	**F**: CAAAATCCAGCACAACAGGAAACGA **R**: CTTGATCTCCAGCCATAATTGGTGG	**638**	

Results and discussion

Bacterial infections cause severe economic losses in poultry industry particularly in developing countries. Infections due to staphylococci are of major importance to veterinary and human medicine ***(El-Jakee et al., 2008).*** In this study we described isolation, identification, antibiotic susceptibility and PCR technique of ***S. aureus*** isolated from apparently healthy newly hatched imported chicks, duckling and poults. Bacteriological examination of 62 imported duck flocks, 52 imported chick flocks and 52 imported poults flocks, revealed that 15 duck flocks from the 62 imported duck flocks with a percentage of 24.2%, 8 chick flocks with a percentage of 15.4% and 14 poult flocks with a percentage of 26.9% were positive for *S. aureus* isolation (Table 4). Similar results nearly obtained by ***Dayamoy and Santosh (2014)*** who recorded that The most frequent staphylococcus infection of veterinary important is *Staphylococcus aureus*, *Staphylococcus pyogenes var albus* and *Staphylococcus pyogenes* and mentioned that out of the 20 duckling sample taken for bacterial isolation and identification, 13 were from Khaki Campbell ducklings and seven were from White Pekin ducklings, all the samples were positive for Staphylococcus aureus isolation. Also, ***Bisgaard (1981)*** isolated 18% *S.aureus* due to arthritis in duck. In contrast to our findings, the results obtained by ***Ismail (2013)*** stated that the percentage of Staphylococci species isolation from duckling in Egypt not exceed 0.9%. Moreover, ***Khalil and El-Shamy (2012)*** reported that the percentage of *S. aureus* isolated from one day old chicks about 20%. In addition to, ***AbdelRahman et al. (2014)*** mentioned that *S. aureus* was present in 29.4% in native and 5.2% in imported chicks. But, ***El-Jakee et al. (2008)*** isolated *S.aureus* from chicks with percentage 8%. Also, ***Linares and Wigle (2001)*** described a case of *S. aureus* pneumonia in turkey poults. Initially, 3-day-old poults with a history of increased mortality were submitted for necropsy. In addition

to, ***Friese et al. (2013)*** who recorded that the prevalence of *S. aureus* on turkey farms with a percentage of 25.9% and this result in line with the results of the national zoonosis monitoring carried out in 2010, which found that 19.6% of turkey farms were positive ***Dombrowski (2012).***

Antimicrobial sensitivity test

As shown in Table (5) Antimicrobial sensitivity test of fifteen *S.aureus* isolates from duckling flocks illustrated that the isolates were highly resistance to Ceftriaxone with percentage 100% then Oxacillin (93.3%), penicillin and Clindamycin (73.3%), Trimethoprim-sulfamethoxazole (53.3%) and Tetracycline (46.7%). While, Amoxicillin + Clavulanic acid showed highly sensitivity 93.3%, then Amikacin, Norfloxacin, Gentamycin, Ciprofloxacin, Doxycyclin, Tetracycline and Ofloxacin with percentage 73.3, 73.3, 60, 60, 60, 46.7 and 40%, respectively But, Erythromycin showing intermediate resistance with percentage 73.3% and Ciprofloxacin 40%. This nearly agreed with ***Mondal and Sahoo (2014)*** who showed 20 Omphalitis cases in ducklings caused by *S.aureus.* The antibiogram showed highly sensitive to Ciprofloxacin and Gentamicin. While, moderately sensitive to Ofloxacin but were resistant to Sulphamethizole. Also**,** ***Persoons et al. (2009)*** showed susceptibility testing for 15 isolated *S. aureus* strains were resistant to erythromycin, tetracycline, and trimethoprim. All strains were susceptible to chloramphenicol, ciprofloxacin. While, ***Neela et al. (2013)*** stated 100% resistance to ciprofloxacin among *S. aureus* on poultry farms in Malaysia and revealed 100% susceptibility towards clindamycin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole and penicillin. On the other hands**,** ***El-Jakee et al. (2008)*** recorded high resistance was among the examined *S. aureus* isolates to amoxycillin, amoxicillin clavulanic acid and gentamicin (66.7% each). Also, ***Ružauskas et al. (2014)*** did not find oxacillin-resistant *S. aureus*.

As shown in Table (6) Antimicrobial sensitivity test of eight *S.aureus* isolates from chick flocks revealed that the isolates were sensitive to Amoxicillin + Clavulanic acid, Amikin, Gentamycin,Ofloxacin, Norfloxacin, Ciprofloxacin, Doxycycline, Penicillin, Tetracycline, Trimethoprim-sulfamethoxazole Clindamycin and Erythromycin by 100%, 87.5%, 87.5%, 75%, 75%, 75%, 50%, 50%, 50%, 37.5% 37.5% and 12.5% respectively. Strains produced intermediate resistance to Doxycycline, Norfloxacin, Ciprofloxacin, Trimethoprim-sulfamethoxazole and Clindamycin by 25% for each. While, Erythromycin, Tetracycline and Gentamycin by 50%, 12.5% and

12.5% respectively. The strains revealed resistance to Oxacillin , Ceftriaxone, Penicillin, Erythromycin, Trimethoprim-sulfamethoxazole, Tetracycline, Doxycycline, Ofloxacin, Amikin, and Clindamycin by 100%, 100%, 50%, 37.5%, 37.5%, 37.5%,25%, 25%, 12.5% and 12.5%. These results is complying with ***Suleiman et al. (2013)*** reported that *S.aureus* strains were susceptible to Ciprofloxacin and Gentamycin but disagree with our study in mentioned that *S.aureus* was resistant to Gentamycin. Higher percent of resistance to Erythromycin and Penicillin has been found which is in accordance with who reported that large proportion of *S.aureus* isolates were resistant to, Penicillin G and Erythromycin ***Daka et al. (2012)***.

In this investigation all *S.aureus* strains were sensitive to Amoxaicillin + Clavinilic acid which agree with ***Losito et al. (2005).***

As shown in Table (7): sensitivity of fourteen *S.aureus* isolates from poult flocks showed that the isolates were highly resistance to Ceftriaxone with percentage 100% then Penicillin (71.4%), Tetracycline (57.1%), Doxycycline and Erythromycin (50%), Clindamycin (35.7%) and Oxacillin (21.4%). While, Ofloxacin and Gentamycin showed highly sensitivity 100%, then Amoxicillin + Clavulanic acid, Norfloxacin, , Ciprofloxacin, Trimethoprim-sulfamethoxazole, Amikacin, Oxacillin , Clindamycin , Doxycycline, Tetracycline and Penicillin with percentage 92.9%, 92.9%, 92.9%, 85.7%, 78.6%, 78.6%, 57.1%, 50%, 42.9% and 28.6%%, respectively. But, Erythromycin showed intermediate resistance with percentage 42.9% and Trimethoprim-sulfamethoxazole, Amikacin 14.3%%. Several workers reported sensitivity and resistance with different antibiotics ***Watts et al. (1993) and Lin et al. (2009).***

Velasco et al. (2014) Stated that similar results obtained from the culture method included a biochemical identification to confirm *S. aureus*, and the results of the conventional multiplex PCR that detected the gene of 16S rRNA. Thirty seven positive strains for *Staphylococcus auresu* represented from examined flocks were subjected to Polymerase chain reaction for confirmation of the isolation results using16S rRNA as common gene for the staphylococci. All the isolates are insured to be staphylococcus. The choice of *clfA* was based on previous work suggesting that *clfA* is present in the chromosome of all *S. aureus* strains ***(*Smeltzer *et al., 1997)***. In addition to ***McDevitt et al. (1994)*** confirmed that the *clfA* gene encodes a surface-exposed fibrinogen-binding protein. In our study when the same isolates examined by *clfA* gene thirty three isolates were *S. aureus* however negative *S. aureus* isolates

were coagulase positive. Similar was stated by ***Velasco et al. (2014)*** that detected three *S. aureus* isolates by PCR instead of that's appeared positive by traditional culture method which concluded that may appear false negative result by PCR. But some investigators like ***El Jaki et al. (2008)*** reported that the production of coagulases and thermonuclease are not unique for *S. aureus* but are shared by other staphylococci. Also, ***Velasco et al. (2014)*** discussed that the improved method of detection of positive *S.aureus* were explained as culturing followed by PCR or PCR from secondary selective enrichment of sample while the PCR from primary selective enrichment of sample or standard culture method alone may lead to high false negative result. While, ***Moussa et al. (2012)*** observed that all the 101 strains (100%) previously identified phenotypically as *S. aureus* with bacteriological examination were positive for 16S rRNA of *S. aureus*.

Table (4) *Incidence of S. aureus isolates in each flock.*

Source of sample	No.of flock	No.of isolates	%* of isolates
Duckling flocks	62	15	24.2%
Chick flocks	52	8	15.4%
Poult flocks	52	14	26.6%
Total	**166**	**37**	**22.3 %**

*Percentage according to the total number of each flock

Table (5) *Results of antibiotic sensitivity test of S. aureus isolated from duckling flocks*

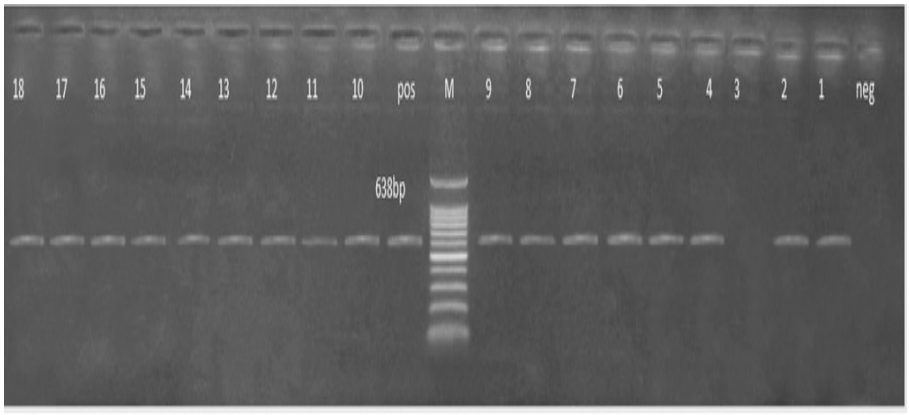
Antimicrobial Discs	Sensitivity of *S. aureus* isolates n = 15					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Amikacin	3	20%	1	6.7%	11	73.3%
Amoxicillin + Clavulinic acid	1	6.7%	0	0%	14	93.3%
Ofloxacin	6	40%	3	20%	6	40%
Clindamycin	11	73.3%	0	0%	4	26.7%
Oxacillin	14	93.3%	0	0%	1	6.7%
Ceftriaxone	15	100%	0	0%	0	0%
Ciprofloxacin.	0	0%	6	40%	9	60%
Doxycycline.	1	6.7%	5	33.3%	9	60%
Erythromycin	3	20%	11	73.3%	1	6.7%
Gentamicin.	4	26.7%	2	13.3%	9	60%
Norfloxacin.	0	0%	4	26.7%	11	73.3%
Penicillin	11	73.3%	0	0%	4	26.7%
Tetracycline.	7	46.7%	1	6.7%	7	46.7%
Trimethoprim-sulfamethoxazole	8	53.3%	5	33.3%	2	13.3%

Table (6) *Results of antibiotic sensitivity test of S. aureus isolated from chick flocks*

Antimicrobial Discs	Sensitivity of *Staph aureus* isolates n = 8					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Amikacin	1	12.5%	0	0%	7	87.5%
Amoxicillin + Clavulinic acid	0	0%	0	0%	8	100%
Ofloxacin	2	25%	0	0%	6	75%
Clindamycin	3	37.5%	2	25%	3	37.5%
Oxacillin	8	100%	0	0%	0	0%
Ceftriaxone	8	100%	0	0%	0	0%
Ciprofloxacin.	0	0%	2	25%	6	75%
Doxycycline.	2	25%	2	25%	4	50%
Erythromycin	3	37.5%	4	50%	1	12.5%
Gentamicin.	0	0%	1	12.5%	7	87.5%
Norfloxacin.	0	0%	2	25%	6	75%
Penicillin	4	50%	0	0%	4	50%
Tetracycline.	3	37.5%	1	12.5%	4	50%
Trimethoprim-sulfamethoxazole	3	37.5%	2	25%	3	37.5%

Table (7) *Results of antibiotic sensitivity test of S.aureus isolated from poult flocks*

Antimicrobial Discs	Sensitivity of *S. aureus* isolates n = 14					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Amikacin	1	7.1%	2	14.3%	11	78.6%
Amoxicillin + Clavulinic acid	1	7.1%	0	0%	13	92.9%
Ofloxacin	0	0%	0	0 %	14	100%
Clindamycin	5	35.7%	1	7.1%	8	57.1%
Oxacillin	3	21.4%	0	0%	11	78.6%
Ceftriaxone	14	100%	0	0%	0	0%
Ciprofloxacin.	0	0%	1	7.1 %	13	92.9%
Doxycycline.	7	50%	0	0%	7	50%
Erythromycin	7	50%	6	42.9%	1	7.1%
Gentamicin.	0	0%	0	0%	14	100%
Norfloxacin.	0	0%	1	7.1%	13	92.9 %
Penicillin	10	71.4%	0	0%	4	28.6%
Tetracycline.	8	57.1%	0	0%	6	42.9%
Trimethoprim-sulfamethoxazole	0	0%	2	14.3 %	12	85.7%



. **Photo (1):** *amplification of the clfA gene of S. auraus for the first eighteen isolates, positive amplification appeared at 638bp lane 1 negative control, lane 11 the positive control and lane 10 the ladder 100+ (Qiagen) .*

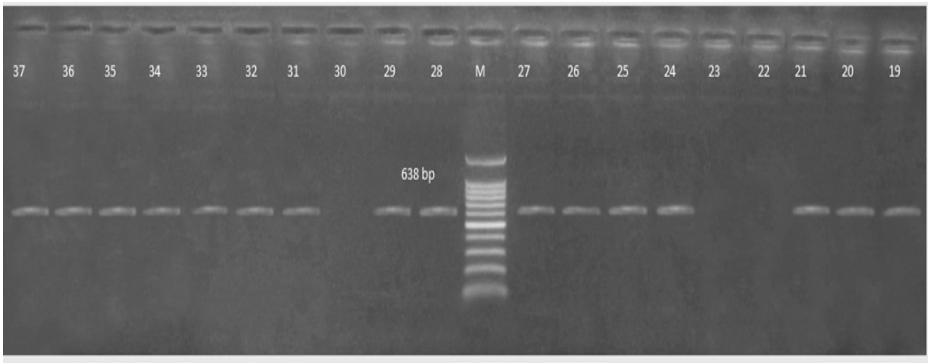


Photo (2): *of the clfA gene of S. auraus for the last nineteen isolates, positive amplification appeared at 638bp , lane 10 the ladder 100+ (Qiagen)*

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Conclusion

Frequent use of antibiotics for treatment of animals and human infections develops resistance. For effective treatment of any staphylococcal infection needs antibiogram. Further investigations should continue to characterize the antibiotic-resistance genes and the epidemiology link between poultry and human. Newly hatched imported chicks, duckling and poults, represent a risk of introducing *S.aureus* to the country. Effective control measures are required to mitigate the economic impact on the poultry industry and to prevent possible public hazards.

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الملخص العربي

تعريف وتوصيف جزيئى لميكروب الاستاف أوريس من الكتاكيت المستوردة حديثة الفقس

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المعمل المرجعى للرقابة البيطرية على الإنتاج الداجنى. معهد بحوث صحة الحيوان. شارع نادى الصيد ص.ب ٢٤٦ - الدقى-١٢٦١٨ - الجيزة- مصر

.تم اجراء الفحص البكتيريولوجى على ١٦٦ قطيع من انواع مختلفة وهى ٥٢،٦٢، ٥٢ قطيع من قطعان البط المستورد و الكتاكيت وكتاكيت الرومى وجد ان ١٥ ، ٨ ،١٤ قطيع من البط و الكتاكيت وكتاكيت ايجابى لعزل ميكروب الاستاف اوريس بنسبة ٢٤,٢٪ ، ١٥,٤٪ و ٢٦,٩٪ على التوالى. وقد تم دراسة مقاومة معزولات الاستاف اوريس للمضادات الحيوية المختلفة. كذلك تم اجراء التوصيف الجزئيى لكل معزولات الاستاف اوريس باستخدام اختبار تفاعل انزيم البلمرة المتسلسل. وهذه الدراسة تلقى النظر على ما تمثله الكتاكيت المستورة من ادخال الاستاف اوريس الى البلد. لذلك فاننا نحتاج الى اجراءات الرقابة الصارمة لتقليل العائد القتصادى على صناعة الدواجن ومنع المخاطر العامة الممكنة.