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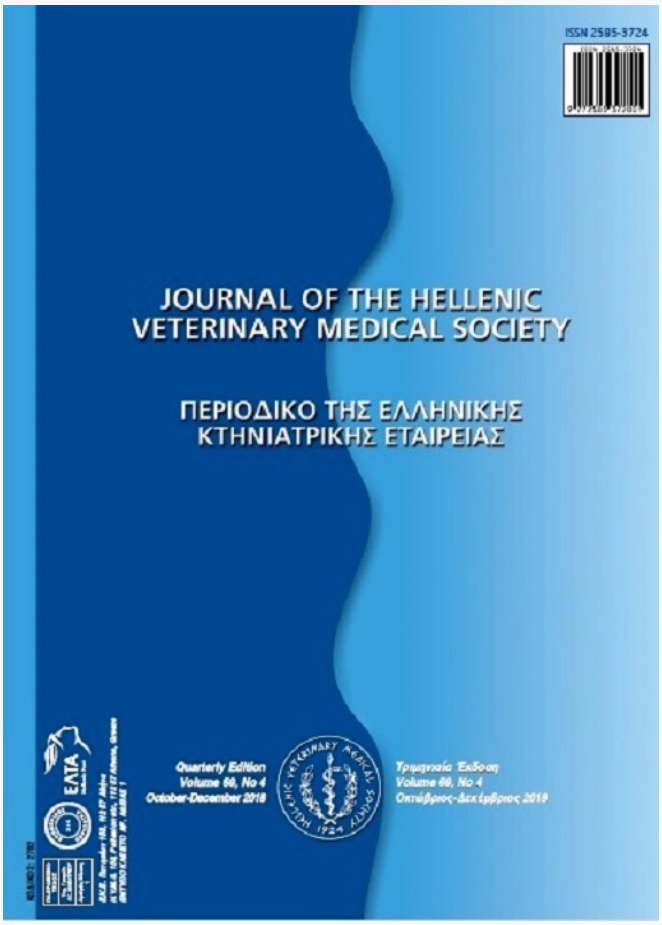

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Reseach article
Ερευνητικό άρθρο

Prevalence and distribution of staphylococcal enterotoxin genes among *Staphylococcus aureus* isolates from chicken and turkey carcasses in Algeria

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ABSTRACT. This study is aimed to determine the prevalence of *staphylococcus aureus* (*S.aureus*) by biochemical tests in poultry carcasses. It is also intend to detect the distribution of genes for classical staphylococcal enterotoxins A, B, C, D and E (*sea, seb, sec, sed* and see) and for gene *femA*, specific for *S.aureus* species, using multiplex PCR.

A total of 385 samples of neck skins from fresh poultry carcasses were collected during the period 2012-2013 from 16 different slaughterhouses located in the region of Algiers, Algeria.

The overall prevalence of *S.aureus* in freshly slaughtered poultry carcasses was 41.56%, with an individual prevalence of 40.63% and 45.71% for chicken and turkey respectively. From the 95 strains of *S.aureus* identified by biochemical tests, 82 (86.32%) isolates were *femA* positive using multiplex PCR. The investigation has also revealed the presence of both enterotoxins B and D, with a predominance of *seb* (13.33%) followed by *sed* (1.67%), in the chicken carcasses while in turkey only *sed* was detected (4.55%)

It has been found that strains of *S.aureus* of poultry origin can be enterotoxigenic with the predominance of genes encoding for enterotoxins *seb* in chicken and *sed* in turkey. As enterotoxins can be produced in adequate amounts to induce foodborne illnesses, these potential dangers must be considered in terms of a real risk to public health.

Keywords: *Staphylococcus aureus*, Poultry carcasses, Staphylococcal entérotoxine, Algiers.

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INTRODUCTION

Recent studies have revealed that high risk of foodborne diseases is often related to the contamination of poultry carcasses by pathogens micro-organisms (Escudero-Gilete et al., 2007). Among the pathogen bacteria that maybe present in poultry carcasses is *Staphylococcus aureus*. Its presence in food indicates poor hygiene and improper storage conditions (Gundogan et al., 2005).

S.aureus is an opportunistic pathogen that can colonize the skin and mucous membranes particularly in the nose of both healthy humans and animals (Nader et al., 2016). In addition to colonizing various hosts, it can also be the origin of a wide range of different infections in poultry ranging from septicemia, pneumonia, endocarditis, and arthritis (Smyth and McNamee, 2008). Amongst the foodborne pathogens *S.aureus* is a major one contaminating meat products. It is considered the third largest cause of food related illnesses throughout the world (Achi and Madubuik, 2007; Aydin et al., 2011; Sasidharan et al., 2011).

S.aureus is characterized by its ability to produce a large variety of Staphylococcal enterotoxins (SEs) (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R and U), but 95 % of food poisoning are caused by theenterotoxins A, B, C, D and E (Letertre et al., 2003). It is notable that SEs are heat stable toxins. Indeed, the heat used in cooking and pasteurization is insufficient to destroy them. Moreover, they are difficult to be perceived in food due to the lack of taste and food appearance (Aycicek et al., 2005). For the case of food ingestion contaminated with SEs, food poisoning occurs shortly after, 30 min to 8 hrs while infected individuals usually recover from the toxicity within 24 - 48 hours(Argudín et al., 2010).

Based on recent published studies, just a little is known about enterotoxigenic *S.aureus* strains from poultry. Particularly in Algeria, the number of studies discussing the prevalence of enterotoxins genes of *S.aureus* in poultry meats is really limited. Therefore, the present study is aimed to determine the prevalence of *staphylococcus aureus* (*S.aureus*) by biochemical tests in poultry carcasses. It is also intend to detect the distribution of genes for classical staphylococcal enterotoxins A, B, C, D and E (*sea, seb, sec, sed* and see) and for gene *femA*, specific *for S. aureus* species, using multiplex PCR.

MATERIALS AND METHODS

Sample collection and microbiological analysis

A total of 385 samples of neck skins from fresh poultry carcasses were collected from 16 different slaughterhouses located in the region of Algiers, Algeria. These samples has been examined during 2012-2013. They consist of chicken carcasses (n=315), and turkey carcasses (n = 70). All samples were placed in sterile plastic bags and brought to the laboratory in cold chain and analyzed within the following 3 h. Microbiological analysis in this study has been carried out according to the international standard NF EN ISO 6888-1/A1(ISO, 2004).Twenty-five portions of the samples were weighed into sterile stomacher bags diluted with 225 ml sterile buffered peptone water (BPW; Oxoid CM 509) and homogenized in a stomacher (Seward 400) for 2 min. The samples were diluted with BPW, and 0.1 ml portions of dilution levels were streaked on Baird-Parker (BP) agar (Oxoid CM 275) supplemented with egg yolk-tellurite emulsion (Oxoid SR 54) and incubated at 37ºC for 24 - 48 h.

The colonies suspected for Staphylococci positive coagulase were cultured on 5% blood agar and identified by catalase, coagulase tests. *S. aureus* was identified by Staphaurex (Bio-Rad). The reference strain used for microbiological analysis was *S.aureus* ATCC 25923. The pure isolates were then stored at −20 °C in TSB-broth and 10% glycerol for future studies.

Statistical Analysis

Data collected from the detection of *S.aureus* on turkey and chicken were analyzed by SPSS Statistics 20 software, to test the significance of differences among the biochemical tests and the Polymerase-Chain Reaction (PCR), and the toxigenic potency of *S.aureus* between the two species (turkey and chicken).

Genomic DNA Extraction

The methodology of DNA extraction is taken fromSambrock and Russel, (2001). For nucleic acid isolation, 95 strains of Staphylococcus species isolated from poultry carcasses were activated on trypticase soya agar (TSA) (BioMérieux, France). After overnight incubation at 37°C, one to two colonies for each strain was re-suspended on 300 µl of TNE buffer (Tris- Nacl-EDTA-SDS) and then vortexed, 10 µl of lysozyme was added plus 200 µl of SETS (Sodium- EDTA- Tris- SDS). The mix was incubated at 37°C for 1 hour. After incubation

Table 1. Oligonucleotide primers sequences used for PCR amplification of *S. aureus's* enterotoxins (SEs) genes.

Gene Primer	Oligonucleotide sequence	Size of amplified product (bp)	
Sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	102
	GSEBR-2	CGGCACTTTTTTCTCTTCGG	
Seb	GSEAR-1	GTATGGTGGTGTAACTGAGC	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG	
Sec	GSEAR-1	AGATGAAGTAGTTGATGTGTATGG	451
	GSEBR-2	CACACTTTTAGAATCAACCG	
Sed	GSEAR-1	CCAATAATAGGAGAAAATAAAAG	278
	GSEBR-2	ATTGGTATTTTTTTTCGTTC	
See	GSEAR-1	AGGTTTTTTCACAGGTCATCC	209
	GSEBR-2	CTTTTTTTTCTTCGGTCAATC	
femA	GSEAR-1	AAAAAAGCACATAACAAGCG	132
	GSEBR-2	GATAAAGAAGAAACCAGCAG	

each tube was vortexed once every 15 min. Then, 10 µl of proteinase- K (Vivantis Technologies, Malaysia) and 100 µl of TNE were added. The final solution was vortexed and incubated at 50°C for 1 h. After that each microcentrifuge tube was vortexed once every 15 min. A volume of 150 µl of NaCl (5M) was added and vortexed. The obtained DNA was harvested by centrifugation at 10.000 rpm for 10 min at room temperature. The supernatant was transferred to a new microcentrifuge tube for washing by 99% cold ethanol and then stored at -20°C overnight. A second washing of DNA was done by 70% cold ethanol. The sample was dried for 10-30 min and re-suspend in 100 µl sterile distilled water and stored at -20°C until PCR analysis.

Multiplex PCR Conditions

The multiplex PCR has been performed as described by Mehrotra et al. (2000). The primers used in the multiplex PCR are presented in Table 2. Multiplex PCR mix contained 400 µM deoxynucleoside triphosphates (dNTPs); 5 µl of 10X reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl); 4 mM $MgCl_2$; 20 pmol of each *sea*, *seb*, *sec*, *see*, and *femA* primers; 40 pmol of *sed* primers; 2.5 U of *Taq* DNA polymerase (AmpliTaq DNA polymerase, Perkin-Elmer), and 5µl of template DNA. The final volume was adjusted by adding 50µl with sterile ultra-pure water. DNA thermocycling (CFX 96thermal cycler, Bio-Rad)was carried out with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 min. Ten microliters of the PCR products were then analyzed by electrophoresis on 2% agarose (BIOMAX) gel, stained with ethidium bromide. Note that the DNA fragments were visualized using a UV transluminator (EC3, UVP Biommaging systems, Inc (BioMax) while immigration was made by Electrophoresis Power Supply (Model EC 1000 XL Thermo Scientific, Inc.) in TBE buffer (0.09 M Tris–HCl, 0.09 M boric acid, 2 mM EDTA, pH 8.3) for 45 min at 120.Primers are shown in Table 1.

RESULTS

Table 2 illustrates the overall prevalence of *S.aureus* in freshly slaughtered poultry carcasses that are collected from 16 slaughterhouses. The prevalence was 160 of

Table 2. Isolation of *S.aureus from* chicken and turkey carcasses

Type of meat sample	**No. of samples**	***S. aureus* positive samples**	
		No.	**%**
Chicken carcasses	315	128	40.63
Turkey carcasses	70	32	45.71
Total	**385**	160	**41.56**

Table 3. Confirmation of the isolated strains as *S.aureus* by multiplex PCR

Origin of the isolates	No. of the isolates	PCR confirmed as *S. aureus* isolates	
		***FemA* positive**	**%**
Chicken carcasses	70	60	85.71
Turkey carcasses	25	22	88.00
Total	**95**	**82**	**86.32**

385 samples (41.56%), with an individual prevalence of (40.63%) and (45.71%) for chicken and turkey respectively.

Table 3 shows the confirmation of the isolated strains as *S.aureus* by multiplex PCR. The multiplex PCR has revealed that 82 (86.32%) from these isolates were *femA* positive, i.e., 60 (85.71%) in chicken and 22 (88%) for turkey. Statistical analysis showed no significant difference between the two methods (Classical method and PCR) ($p< 0.05$).

It has also been observed that 12.19% of *S.aureus* isolates encoded classical staphylococcal enterotoxins, i.e., nine (15%) from chicken and 1 (4%) from turkey carcasses. Statistical analysis showed no significant difference for the prevalence of enterotoxigenic isolates from chicken carcasses and turkey for $p< 0.05$.Moreover, detected the presence of both enterotoxins B and D in poultry carcasses with a prevalence of 9.76% and 2.44% respectively.

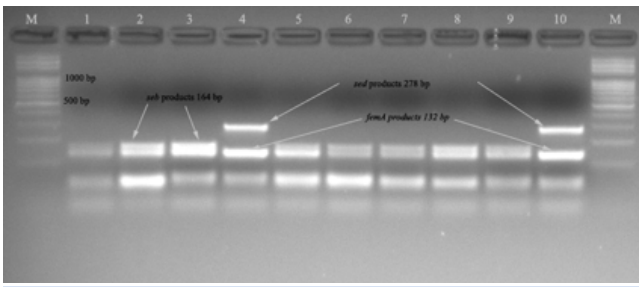

Figure1. Agarose gel electrophoresis patterns showing multiplex PCR amplification. Lanes M represent100bp DNA ladder . Lanes 1 to 7 represent, PCR amplicons from primer set A. Lanes 8 to 10 represent PCR amplicons from setB. Lanes: 1,2,3,5,6,7,8 and 9 present the characteristic band of *seb while* 4 and 10 present the characteristic band of ,*sed.*In all cases the band *femA* is present.

As indicates in Figure 1 and Table 4, isolates from chicken present both enterotoxins B and D, with a predominance of *seb* (13.33%) followed by *sed* (1.67%), while in turkey only *sed* was detected (4.55 %). It is notable that none of the isolates was positive for *sea*, *sec* or *see* genes.

DISCUSSION

The isolation of *S.aureus* from chicken and turkey carcasses (41.56%) confirms the contamination of the poultry carcasses by *Staphylococcus aureus*. Indeed, the present study is mainly focused on the determination of the prevalence of *S.aureus* in freshly slaughtered chicken and turkey, in 16 slaughterhouses in the Wilaya of Algiers. Moreover, it also aims to the molecular characterization of the isolated strains by highlighting the presence of enterotoxins genes.

The prevalence of *S.aureus* recorded for chicken (40.63%) was in good agreement with those advocated

Table 4. Distribution of *Staphylococcus aureus* enterotoxins genes in poultry carcasses

Origin of isolates	Screened isolates	Se positive (%)	Types of entero-toxin' genes(%)				
			Sea	***Seb***	***Sec***	***Sed***	***See***
Chicken carcasses	**60**	**9 (15.0)**	**0 (0)**	**8 (13.33)**	**0 (0)**	**1 (1.67)**	**0 (0)**
Turkey carcasses	**22**	**1 (4.55)**	**0 (0)**	**0 (0)**	**0 (0)**	**1 (4.55)**	**0 (0)**
Total	**82**	**10 (12.19)**	**0 (0)**	**8 (9.76)**	**0 (0)**	**2 (2.44)**	**0 (0)**

by Citak and Duman (2011) and Nader et al.(2016). In Algeria a study conducted in the region of Biskra, carried out on 60 chicken carcasses revealed a prevalence similar to that obtained in the present study (Alloui et al., 2013). However, lower prevalence rates of *S.aureus* were found by others studies (Khallaf et al., 2014; Sarrafzadeh Zargar et al., 2014).

In our study, the recorded prevalence of *S.aureus* present in turkey carcasses (45.71%) was higher than that obtained by Sarrafzadeh Zargar et al.(2014) (16.6%) and El Allaoui (2013) (41.6%).

Two other studies that have been carried out in Turkey recorded a prevalence of 9.61% (Kiliç et al., 2009) and 48% (Bystron et al., 2005) of coagulase-positive *Staphylococcus* from meat and hash meat respectively.

The high prevalence recorded in both species often indicates a lack of good hygiene practices(Lindblad et al., 2006; Pacholewicz et al., 2016) which are linked to the multiple handling of carcasses during slaughter processing. The staff represents a source of contamination spreading the germs by inadequate clothing hygiene, or by hosting (sores, angina, sinusitis and nasopharyngitis).

The lack of control of good manufacturing practices can also be at the origin of these contaminations-multiple contacts with the equipment: tables, bags, knives, towels, scalding bath and feathery (Azelmad et al., 2017; Kotula and Pandya, 1995;Mead et al., 1993).

It could also be related to the sanitary status of poultry intended for slaughter (carried by the skin and feathers, or various pathologies as osteomyelitis, arthritis and synovitis).

The variations in the prevalence between our results and other studies, that have been carried-out in other countries, can be attributed to several factors, including the size of the samples tested, the sampling mode(Whole chicken or parts of carcasses), the seasons in which the sampling was done, the methods of isolation used and the hygiene conditions of the slaughterhouse(Teramoto et al., 2016; Wang et al., 2013).

The use of PCR and biochemical identification to confirm *S.aureus* strains revealed similar results (average86.32%), which confirms the concordance between the two techniques, already reported by the study ofBenhamed (2014).

The present study shows that the poultry isolates can be enterotoxigenic (12.19%). These results are in good agreement with the previous published studies which revealed that the percentage of *S.aureus* strains producing enterotoxin A to E, bovine and avian, can vary from 0 to 15% (Bergdoll, 1991; Genigeorgis, 1989;Rosec et al., 1997). More recent studies showed that the genes encoding the classical enterotoxins, were absent or occur in less than 3% in *S.aureus* strains isolated from poultry (Hazariwala et al., 2002; Normanno et al., 2007; Smyth et al., 2005).

As for the chicken some authors note relatively higher percentages. For instance, Nader et al.(2016)show that 7 strains out of 12 (58.3%) of *S.aureus* isolated from chicken meat were enterotoxigenic.They also reported the presence of *sea*, *seb* and *sed* genes in chicken meat isolates, with percentages of 33.3%; 8.3% and 16.7% respectively.

Kitai et al.(2005) revealed that 21.7% of *S.aureus* strains, isolated from chicken carcasses were enterotoxigenic. Moreover, Nemati (2013) observed that71% of *S.aureus* strains isolated from chicken nose and cloaca were enterotoxigenic. As for the turkey isolates, Koluman et al.(2011) showed that the production of enterotoxins in turkey meat was 36%.

However, the present study demonstrated that the prevalence of enterotoxigenic isolates from chicken carcasses (15%) and turkey (4.54%) is relatively lower.

Although, there was a variability in the frequency of different enterotoxins types production, in chicken we observed the dominance of *seb* gene with a rate of 13.33% compared to the *sed gene* (1.67%).Similar results were stated by Kitai et al.(2005). Nevertheless, only type D enterotoxins (4.54%) were detected in turkey isolates. This variability between the two species had not yet been defined.

Madahi et al.(2014) showed that *S.aureus* strains isolated from chicken nuggets present staphylococcus enterotoxins genes, with values of 33.33%; 4.16%; 12.50%; 8.33%, 12.50%; 12.50% for *sea*, *seb*, *sec*, *sed*, *sea* + *sec* and *sea* + *sed* respectively. No *see* gene was detected. Another study by Nemati (2013) also reported that 33.3% of *S.aureus* strains isolated from the nose and cloaca of healthy chicken were positive for *sea* and 5% had a mixed *sea* + *egc* enterotoxins production, whereas the genes encoding *seb*, *sec*, *sed* and *see* were absent.

A study of the incidence of enterotoxigenic *S.aureus* strains in turkey meat, was carried out by Bystron et

al.(2005) presenting that from the 4 coagulase-positive Staphylococcus strains, isolated from 11 samples of turkey meat the 3 strains had enterotoxin genes of type B and one had type C. Adams and Mead (1983)isolated enterotoxigenic Staphylococci in 2 of the 3 turkey' slaughterhouses studied. They reported that in the first slaughterhouse60% of the 55 isolates produced enterotoxin C while in slaughterhouse B, 4% of the 41 isolates produced enterotoxin D and 2% produced enterotoxin F. No enterotoxigenicity was detected from the 50 isolates in the third slaughterhouse.

As type A enterotoxin is usually typical for the human origin isolates (Orden et al., 1992) and type C enterotoxin is frequently produced by bovine origin isolates (da Silva et al., 2005; Jorgensen et al., 2005; Katsuda et al., 2005)could explain their absence in poultry studies.

The variability observed in the frequency of enterotoxin production by *S.aureus* strains may be related to the origin of the studied isolates (food or other) and their geographic origin(Bergdoll, 1991; Genigeorgis, 1989; Larsen et al., 2000; Rosec et al., 1997).

Type A, B and D enterotoxins are implicated in 95% of reported foodborne illness outbreaks (Letertre et al., 2003). During the years 2001-2003 in Taiwan, enterotoxins A, B, C, and D were detected with respective percentages of 29.2%; 19.7%; 6.8% and 2.0% in patients associated with staphylococcal foodborne illness outbreaks (Chiang et al., 2008). Furthermore, more than 50% of staphylococcal food-borne infections were caused by type A enterotoxin. In addition, *sea* and *seb* enterotoxins are the two most important agents causing gastroenteritis, they are also the most implicated in foodborne illness (> 60%) in the United States and England (Kluytmans and Wertheim, 2005).

CONCLUSION

We have experimentally determined the prevalence and enterotoxins genes of *S.aureus* in fresh poultry carcasses that are collected from 16different slaughterhouses located in the region of Algiers, Algeria. The investigation has revealed that the microbial risk by *S.aureus* in poultry carcasses is not negligible. It has been found that the prevalence of *S.aureus* in 385 carcasses was 41.56% with an individual prevalence of 40.63% and 45.71% for chicken and turkey respectively. The strains of *S.aureus* of avian origin can be enterotoxigenic with the predominance of the genes encoding for enterotoxin *seb* in chicken and *sed* in turkey.

When meat is preserved under favorable conditions for germs multiplication and toxinogenese, enterotoxins can be produced in sufficient amount to trigger foodborne illnesses. These potential dangers must be considered in terms of real risk to public health. To ensure the safety and hygienic quality of meat, the application of good hygiene practice (GHP) and the implementation of HACCP in poultry slaughterhouses has become an absolute necessity to protect consumer' health.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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