

DETECTION OF *Staphylococcus aureus* FROM LOCAL AND IMPORTED CHICKEN IN DUHOK PROVINCE/KURDISTAN REGION OF IRAQ USING CONVENTIONAL AND MOLECULAR METHODS

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ABSTRACT

This study aimed to detect *Staphylococcus aureus* in local fresh whole chicken and frozen imported chicken using conventional and PCR assay by targeting specific *S. aureus* thermonuclease gene (*nuc*). A total of 200 whole chicken carcasses were examined and samples include 100 of local chickens from the Duhok chicken abattoir and 100 of imported chicken from supermarkets in Duhok city. The samples were cultured on mannitol salt agar and the confirmation is done by colony morphology, Gram stain, biochemical test including catalase test and coagulase test. The results showed that 28 (28%) of 100 local chicken and 80 (80%) of 100 imported frozen chicken carcasses were found to be positive with *S. aureus* using conventional methods. Amplification of *nuc* gene specific for *S. aureus* is used to confirm the isolates of *S. aureus*. The results showed that only 18 of 22 coagulase positive isolates from local chicken and 57 of 68 coagulase positive isolates from imported chicken were confirmed as *S. aureus*. The results indicate that PCR assay seem to be more specific for detection of *S. aureus* in food sample and appear to be more reliable than conventional methods for assessing bacteriological safety of food. These results showed high prevalence of *S. aureus* in imported chicken than in local chicken meat and thus may happen as a result of processing and storage conditions.

INTRODUCTION

In developing countries, food-borne diseases (FBD) are one of the major concerns causing annually several deaths along with economic losses. According to World Health Organization (WHO), annually an estimated 600 million people are infected and about 420,000 die worldwide due to ingestion of food contaminated with foodborne pathogens. Many pathogens, including *E. coli*, *Salmonella*, *Campylobacter*, *Listeria*, *Vibrio cholera* and *S. aureus* have been reported to be responsible for food-borne outbreaks around the world⁽¹⁾. Worldwide *S. aureus* food-borne is one of the major concerns in public health^(2, 3, 4, 5). *S. aureus* causes various infections including skin infections to severe invasive disease^(4, 6). *S. aureus* can grow in a wide range of temperatures but the optimum is 30 to 37 °C, optimum pH 7 to 7.5 and at NaCl concentrations as high as 16%⁽⁷⁾. There are some characteristics of the *S. aureus* that favour its growth in many food products⁽⁸⁾. These features include surviving in dry and stressful environments. For example, nose, on skin, and inanimate surfaces⁽⁹⁾. Therefore, it can remain persist for extended duration after initial contact on hands and environmental surfaces⁽¹⁰⁾. Food poisoning by *S. aureus* occurs rapidly following consumption of contaminated food and it usually takes 3 to 5 hours. This is due to the production of toxins by *S. aureus* as they grow at optimum temperature⁽⁸⁾. For *S. aureus*, variety of foods provides as an optimum growth medium. It has been reported that variety of foods including meat and meat products, poultry meat and poultry products and, milk and dairy products are frequently involved in *S. aureus* FBD^(3, 8, 11, 12, 13, 14, 15). Other major factors that are involved in increasing the number of FBDs are absences of sanitary conditions and incorrect microbiological checking of food⁽⁵⁾.

Furthermore, other conditions are involved to a high number of FBD outbreaks especially in retail food industry such as improper handling processes of food⁽¹⁶⁾. Poultry meat is one of the popular foods with constant increase in consumption worldwide and commonly contaminated by antibiotic resistant *S. aureus* strains^(5, 17). *S. aureus* is found normally on the skin and in the intestinal epithelium of animals and therefore, during slaughtering process this may contaminate meat. Possibly contamination of meat may also happen during meat processing and storage time because this pathogen is widely distributed in environment. Consequently, one of the main cause of FBD in human around the world is ingestion of contaminated raw poultry meat^(5,17) because of above information that emphasises the importance of detection of *S. aureus* in whole raw chicken as major

foodborne pathogen in human. Therefore, the aim of this study was to detect *S. aureus* from local whole raw chicken obtained from Duhok chicken slaughter house in a Summel and whole raw imported chicken from supermarkets in Duhok city using both conventional and molecular methods.

MATERIALS AND METHODS

Sample collection:In this study, a total of 200 whole chickens were examined for the detection of *S. aureus*. The samples include 100 of local chickens and 100 of imported chickens. Local raw chicken carcasses were collected from the Duhok chicken abattoir in Summel. The whole imported chicken carcasses were collected from supermarkets in Duhok city. Both local and imported chicken were collected and placed in a cold box and transported immediately to Microbiology Laboratory at College of Veterinary Medicine for microbiological analysis.

Identification of bacteria using conventional cultural methods: The whole chicken including both local and imported was transferred to a sterile plastic bag and 400 ml buffer peptone water (BPW) was added to each bag. The whole chicken was then rinsed inside and out for 2 min with a rocking motion. Aseptically the whole chicken was removed from the bag, and the rinse was collected. For isolation 10 ml of chicken rinse was collected aseptically from fresh local chicken and pipetted into 90 ml of BPW. The broths were incubated for 24 h at 37°C. After 24 h incubation, a loop from the broth was cultured onto the mannitol salt agar (MSA). Plates were incubated for 18-24 h at 37°C. *S. aureus* colonies were first identified according to Bergeys Manual of systematic Bacteriology⁽¹⁸⁾ based on colonial morphology, Gram-stain and biochemical tests. The suspected *S. aureus* colonies were selected then streaked onto MSA to obtain pure colonies of *S. aureus*. Colonies with typical morphology were selected and subjected to Gram-staining and catalase test. These isolates were further examined using coagulase test and molecular method. Phenotypically suspected *S. aureus* isolates were purified and maintained in 50% glycerol and brain heart infusion broth (BHIB) stocks at -20°C for further processing.

Molecular detection: Phenotypically suspected *S. aureus* isolates were further confirmed by PCR.

DNA extraction: *S. aureus* isolates were plated out onto MSA from -20°C stock cultures and incubated overnight at 37°C for 24 h. Chromosomal DNA was prepared according ⁽¹⁹⁾. Briefly, DNA was prepared by resuspending 3 to 4 bacterial colonies in 50 µl of deionized doubled distilled water and mixed very well. Bacterial suspension was boiled in water bath for 5 to 10 min. The suspension was then centrifuged at 10,000 x g for 1 min. The supernatant was collected and used for PCR as the DNA template. NanoDrop 2000C spectrophotometer (Thermo Scientific) was used to check DNA purity and concentration. The DNA samples were stored at -20°C.

Primers design for amplification of *nuc* gene : The thermostable nuclease gene (*nuc*) was selected for detection of *S. aureus* (*nuc*, *S. aureus* specific). *S. aureus* strain NCTC5660 complete genome (accession number: LR134088) was used as a reference genome to locate the *nuc* gene. Blast analysis was used to identify the position of the *nuc* gene and it was located at position 821400 to 821959 in the NCTC5660 genome. *Nuc* gene is annotated as a thermonuclease (locus tag: NCTC5660_00914). BLAST analysis of *nuc* gene against the National Centre for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/genome/genomes/154>) was carried out to find the identities of the *nuc* gene in *S. aureus* genomes. The sequences of *nuc* gene identities were aligned using the BioEdit sequence alignment editor software. The consensus sequence was formed from the aligned sequences and it was used to design primers for the detection of *S. aureus* by PCR assay. The primers were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and they were synthesised by Macrogen (South Korea). Details of the oligonucleotide primers are listed in Table 1.

Table 1: Details of oligonucleotide primers used for amplification of *nuc* gene (*S. aureus* specific).

Primer name	Sequence (5'—3')	Length	Product size (bp)	Ref.
nuc-F1	AGCGATTGATGGTGATACGG	20	226	This study
nuc-R1	ATACGCTAAGCCACGTCCAT	20		This study
nuc-F2	AAGCGATTGATGGTGATACGG	21	281	This study
nuc-R2	AGCCAAGCCTTGACGAACT	19		This study

Amplification of *nuc* gene by PCR The *nuc* gene was amplified using ready-to-use mixes for PCR, Ruby *Taq* Master (Jena Bioscience, Thuringia, Germany). According to the manufacturer's instructions, PCR assays were carried out using a total reaction volume of 20 μ l. Each reaction consisted of the following reagents: 10 μ l of master mix, 1 μ l of each forward and reverse primer (10pml/ul), 2 μ l of template DNA (50ng/ul) and 6 μ l dH₂O. Gene Amp PCR System 9700 Thermo Cycler (Applied Biosystems) was used to perform PCR amplification.

The following PCR parameters of 35 cycle reactions were used, initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 55°C for 0.5 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Confirmation of amplified *nuc* gene was performed using a 1% agarose gel electrophoresis prepared with TAE (1X) buffer containing Prime Safe Dye (GeNet Bio, Korea). Five microliters of each PCR product were loaded. 5 μ l of 100bp DNA ladder (GeNet Bio, Korea) was loaded to check the size of amplified PCR product. Finally, UV light was used to visualize and photograph the fragment sizes. The amplified products were stored at -20°C for future analysis.

RESULTS

Prevalence of *S. aureus*:

A total of 200 whole chicken carcasses were examined and these samples include 100 of local chickens from the Duhok chicken abattoir and 100 of imported chicken carcasses from supermarkets in Duhok city. The prevalence of *S. aureus* on the local and imported chicken carcasses using conventional method was found to be 108/200 (54%). The results showed that the prevalence *S. aureus* in local chicken was lower than in imported chicken. Overall 28/100 (28%) of local chicken and 80/100 (80%) of imported chicken carcasses were carried *S. aureus* by conventional method as listed in Table 2.

Table 2: Prevalence of *S. aureus* isolated from Local and imported carcasses using conventional methods

	No. of samples	No. of Positive samples	Prevalence of <i>S. aureus</i> (%)	No. of coagulase positive (%)	No. of coagulase negative (%)
Local Chicken	100	28	(28)	22 (78.5%)	6 (21.5%)
Imported Chicken	100	80	(80)	68 (85%)	12 (15%)
Total	200	108	(54)	90 (83.3%)	18 (16.7%)

Isolation of *S. aureus* using conventional culture methods

The overall results using conventional culture methods from both local and imported chicken carcasses are presented in Table 2. Presumptive isolation of *S. aureus* was carried out on MSA and the primary identification was performed based on the unique characteristic of *S. aureus* on MSA which appears as yellow colonies with yellow background as a result of mannitol fermentation. Out of 200 samples inoculated on MSA plates using streak plate method, 108 isolates (28 of 100 local chicken and 80 of 100 imported frozen chicken carcasses) showed yellow colonies with mannitol fermentation (Table 2). Mannitol fermenter colonies on MSA were further sub-cultured in order to be characterized by microscope and biochemical tests.

Identification of *S. aureus*:

Mannitol fermenter colonies were exposed to Gram stain and biochemical tests such as catalase test, coagulase test for the characterization of isolates. All the 108 mannitol fermenter isolates including 28 from local chicken and 80 from imported frozen chicken carcasses appeared as Gram positive grape-like cocci and catalase positive. Bacterial isolates were further analysed using coagulase test. In this study, 22 of 28 (78.5%) of local chicken carcasses isolates and 68 of 80 (85%) of imported chicken carcasses isolates were found to be coagulase positive. However, 6 of 28 (21.5%) of local chicken isolates and 12 of 80 (15%) of imported chicken isolates were found to be coagulase negative using both tube and slide coagulase test (Table 2).

Identification of *S. aureus* based on *nuc* gene by PCR

Two sets of specific primers were synthesized for this study and these primers were targeting the *nuc* gene (*nuc*, *S. aureus* specific) as shown already in material and methods. Previously, it has been revealed that rapid diagnosis of *S. aureus* depends on the amplification of the *nuc* gene (20). Identification of *S. aureus* strain was based on 226 bp of *nuc*-F1 and *nuc*-R1 and 281 bp of *nuc*-F2 and *nuc*-R2 as shown in Fig. 1, amplified products were similar to the sequence of the *nuc* gene on 1% agarose gel. Amplification of *nuc* gene fragment by PCR showed that only 81.8% (18 of 22) coagulase positive isolates from local chicken and 83.8% (57 of 68) coagulase positive isolates from imported chicken were confirmed as *S. aureus*. Overall 83.3% (75 of 90) of coagulase positive isolates from both local chicken carcasses and from imported chicken carcasses were confirmed as *S. aureus*.

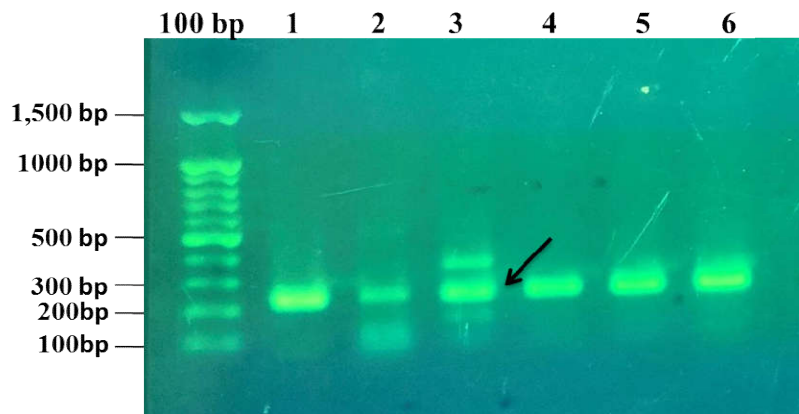


Fig. 1: Amplified products of the *nuc* gene in coagulase positive *S. aureus* isolated from local and imported chickens in 1% agarose gel electrophoresis. Lane 100bp: molecular size marker. Lanes 1-6: amplified *nuc* gene in *S. aureus* isolates

DISCUSSION

One of the major public health concerns nowadays is microbial food safety because of its major influence on food. Poultry meat consumption is increasing worldwide and this result from high value in protein and nutrient contents^(5, 17). For that, it is very important to ensure the microbial safety and proper hygiene of both poultry meat in order to prevent contamination of meat that may be dangerous to consumers^(4, 17, 21, 22). Chicken meat and other poultry products are usually contaminated by many bacteria such as *E. coli*, *Salmonella*, *S. aureus*, and *C. jejuni* etc. *S. aureus* is among the common food contaminants and able to produce enterotoxins (SEs) that cause varying degree of intoxication in humans through

ingestion of contaminated food ^(23, 24). The source of outbreaks can be detected through isolation of *S. aureus* in poultry meat ⁽²⁵⁾. Outbreaks of *S. aureus* FBD is related with the consumption of poultry meat and mostly improper cooked meat, poor microbiological safety and storage conditions of meat and meat products. Generally, cultural methods are used for the isolation and identification of *S. aureus* from chicken meat as mentioned above in materials and methods. In this study, colony morphology on MSA, Gram stain, catalase and coagulase test are used for the primary identification of *S. aureus* in chicken sample ^(26, 27, 28, 29, 30).

In this study, a total of 200 whole chicken carcasses including both local and imported chicken were examined, 108 of them showed unique characteristic of *S. aureus* on MSA which appears as yellow colonies. Primary confirmation was performed based on some biochemical test. All 108 isolates were appeared to be grape-like Gram positive cocci by Gram staining and they were catalase positive ⁽³¹⁾. Generally, coagulase test is one of the tests which is used to confirm identification of *S. aureus*. Therefore, 108 bacterial isolates were further tested by coagulase test. In this study, 22 of 28 (78.5%) of local chicken isolates and 68 of 80 (85%) of imported chicken isolates were found to be coagulase positive. However, 6 of 28 (21.5%) of local chicken isolates and 12 of 80 (15%) of imported chicken isolates were found to be coagulase negative using both tube and slide coagulase test. Previously, it has been shown that out of 150 samples 98 samples (68.53%) were coagulase positive and 22 samples (31.46%) were coagulase negative ⁽²⁵⁾. In another study, it also has been found that out of 195 samples examined, 92 (47.2%) were coagulase positive and 103 (52.8%) of 195 were coagulase negative ⁽³²⁾. However, other species of *Staphylococcus* including *S. intermedius* and *S. hyicus* are coagulase positive ⁽³³⁾. It has been revealed that among 487 coagulase positive *Staphylococcus*, 82.1%, 17.7% and 0.2% were *S. aureus*, *S. hyicus* and *S. intermedius*, respectively ⁽³⁴⁾.

Therefore, primers targeting specific *S. aureus nuc* gene were designed and amplified by PCR for definitive confirmation of *S. aureus*. The overall infection of *S. aureus* on the local and imported chicken carcasses using conventional method was found to be 54%. The results showed that the occurrence of *S. aureus* in local chicken was lower than in imported chicken. Overall 28% of local chicken and 80% of imported chicken carcasses was found to be infected with *S. aureus* using conventional method. The rate of contamination and infection in this study was different when it is compared with other studies. The variation in

the percentage of *S. aureus* infection in chicken meat suggests that contamination of meat could be due to handling, processing, transportation and storage⁽³⁵⁾. As mentioned above that other *Staphylococcus* species including *S. intermedius* and *S. hyicus* are coagulase positive⁽³³⁾. Therefore, molecular diagnostic methods such as PCR assay are used for better differentiation of pathogens in terms of species level^(15, 36, 37). Previously, it has been shown that rapid diagnosis of *S. aureus* infections can be done through PCR amplification of the *nuc* gene⁽²⁰⁾. Amplification of *nuc* gene fragment by PCR showed that only 81.8% (18 of 22) coagulase positive isolates from local chicken and 83.8% (57 of 68) coagulase positive isolates from imported chicken were confirmed as *S. aureus*. Overall 83.3% (75 of 90) of coagulase positive isolates from both local chicken and from imported chicken were confirmed as *S. aureus*.

The data of this study showed the differences between conventional and PCR for the identification of *S. aureus* from chicken meat. This confirms that PCR assay is specific to diagnose and to understand the mechanisms involved in pathogenesis, resistance and survival of the strains in raw meat⁽³⁷⁾. Overall, the presence of *S. aureus* is normally not grounds for rejecting carcasses. However, it is often considered an indicator of the overall hygienic status of slaughter plants⁽³⁸⁾. Proper management, hygiene and microbial safety of poultry meat are highly recommended especially in supermarkets in order to minimize the occurrence of food-borne diseases including *S. aureus* food-borne from either fresh or frozen chicken meat to human. Generally, contamination of carcasses, cuts, and processed meat products occur through bacteria from animal intestine, environment of slaughterhouse and the equipment that are used during and after slaughtering⁽¹⁷⁾. Some of these bacterial contaminants are resistant and are capable to grow and survive especially during processing and storage of food⁽¹⁷⁾. Subsequently, proper management, hygiene and microbial safety of poultry meat is highly recommended either fresh or frozen chicken meat in order to minimize the occurrence of food-borne diseases including *S. aureus* food-borne in human.

تشخيص بكتريا المكورات العنقودية الذهبية المعزولة من الدجاج المحلي والمستورد في مدينة دهوك – كوردستان العراق باستخدام الطرق التقليدية والجزئية

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الخلاصة

هذه الدراسة هدفت الى عزل وتشخيص بكتريا المكورات العنقودية الذهبية من عينات لحوم دواجن محلية ومن عينات لحوم مجمدة مستوردة باستخدام طرق التشخيص الاعتيادية والجزئية مثل تفاعل سلسلة البلمرة Polymerase Chain Reaction (PCR) لجين (*nuc*) الخاص بعزلات المكورات العنقودية الذهبية . تم فحص 200 عينة لحوم دواجن كاملة بواقع 100 عينة لحوم دواجن مذبوحة في مجازر مدينة دهوك و 100 عينة لحوم دواجن مستوردة مجهزة من محلات في مدينة دهوك . تم زراعة العينات على أوساط المانيتول الملحي ليتم تأكيد شكل المستعمرات وأجراء فحص صبغة غرام ، فحص الكتاليز وتخثر بلازما الدم . اكدت النتائج باستخدام طرق التشخيص التقليدية ان 28 (28%) من عينات اللحوم المحلية و 80 (80%) من عينات الحوم المجمدة المستوردة كانت ملوثة ببكتريا المكورات العنقودية الذهبية . تم استخدام تقنية تفاعل سلسلة البلمرة PCR لجين (*nuc*) لغرض تأكيد نتائج التشخيص التقليدي السابقة حيث اظهرت النتائج انه فقط 18 من اصل 22 عينة محلية كانت موجبة لفحص تخثر بلازما الدم و 57 من اصل 68 عينة لحوم مستوردة تم تأكيدها على انها بكتريا المكورات العنقودية الذهبية . لذا فان هذه النتائج تشير الى ان تقنية تفاعل سلسلة البلمرة PCR هي اكثر حساسية وخصوصية لتشخيص بكتريا المكورات العنقودية الذهبية في عينات الغذاء وكما تشير أيضا الى ان هذه التقنية هي اكثر مصداقية من الطرق التشخيصية التقليدية في تقييم السلامة الجرثومية لعينات الغذاء . وأخيرا فان نتائج هذه الدراسة تشير الى ان التلوث ببكتريا المكورات العنقودية الذهبية قد يكون أكثر شيوعا في عينات لحوم الدواجن المستوردة مما هو عليه في عينات اللحوم المذبوحة محليا وقد يكون السبب هو التلوث خلال عمليات تحضير وخن اللحم المستوردة.

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