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Isolation and molecular characterization of staphylococcus aureus isolated from clinical cases in broilers

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Abstract

Background: Staphylococcus aureus (*S. aureus*) causes a difficult problem in the poultry industry because it causes diseases that are difficult to treat due to the resistance of these bacteria to antibiotics and their possession of a battery of virulence and resistance genes in addition to their ability to produce thick biofilms.

Method: A cross-sectional study conducted to collect a total of 53 samples from different clinical cases in broilers during the period from August 2019 to February 2020 in Al-Najaf and Karbala cities, The clinical isolates were determined by using the conventional standard biochemical tests. All the specimens cultured on blood agar medium supplemented with 5% blood for primary isolation and selected by using selective media mannitol salt agar (MSA) for confirmation the mannitol fermentation, then subjected to gram's staining, catalase, oxidase, and further slide coagulase test, then all *S. aureus* isolates tested by antibiotic susceptibility test, and screened for the presence of mecA and mecC genes using PCR for the detection of MRSA isolates, then subjected to the detection of virulence genes (pvl and eta), antibiotic resistance gene (cfr), identification of integron class 1, biofilm formation assay, the multi-druge resistance profiles (MDR) and multible antibiotics resistance (MAR) indexes were calculated.

Results: the isolation rate of *S. aureus* from the broilers' clinical samples was 37.7%. The antibiotic susceptibility test revealed that 85% of *S. aureus* isolates were resistant to one or more of the antibiotic tested. All 53 isolates were assessed for the presence of mecA and mecC genes by using PCR. The mecA gene-specific PCR product was seen in 7 (35%) isolates and considered as MRSA. Among all S. aureus isolates, two isolates were positive for the eta gene, and 15 (75%) isolates harboring integron class 1, while the biofilm formation test revealed that 7 (35%) was positive biofilm producers and three of them were strong producers, consequentlly, 13 (65%) of the isolates were resisted to three or more antibiotics and considered as MDR strains. While pvl, cfr, and mecC gene were not detected among S. aureus isolates.

Conclusion: the current study revealed that *S. aureus* possess a real threat in the poultry industry reflecting a public health problem due to the large acquisition of antibiotic resistance genes by these bacteria, the results indicated a high percentage of isolates having MDR characteristic, and two of them were resistant to all antibiotics tested. In addition to the

presence of two MRSA isolates carrying the eta gene, this indicating that they are of human origin.

Keywords: MRSA, S. aureus, mecA gene, mecC, pvl, eta, cfr , MDR, MAR.

توصيف المكورات العنقودية الذهبية المقاومة للميثيسيلين المعزولة من عينات حليب البقر والدواجن

انس اسماعيل الموسوي عبدالله عبيس الحاتمي

الخلاصة:

الخلفية: تسبب بكتيريا Staphylococcus aureus مشكلة صعبة في صناعة الدواجن لأنها تسبب أمراضًا يصعب علاجها بسبب مقاومة هذه البكتيريا للمضادات الحيوية وامتلاكها لمجموعة من الجينات الضراوة والمقاومة بالإضافة إلى قدرتها على انتاج الأغشية الحيوية السميكة.

الطريقة: تم جمع 53 عينة من الحالات السريرية المختلفة في دجاج اللحم خلال الفترة من آب 2019 إلى شباط 2020 في مدينتي النجف وكربلاء ، وتم تحديد العزلات السريرية باستخدام الاختبارات البيولوجية والكيميائية القياسية التقليدية. تمت زراعة جميع العينات على وسط أجار الدم المضاف إليه 5٪ من الدم للعزل الأولي واختيارها باستخدام وسائط انتقائية (MSA) لتأكيد تخمير المانيتول ، ثم تعريضها لتلطيخ الجرام ، catalase ، oxidase ، و slide coagulase، بعد ذلك تم عمل اختبار جميع العزلات عن طريق اختبار الحساسية للمضادات الحيوية ، وتم فحصها بحثًا عن وجود جينات mecA و mecC باستخدام PCR للكشف عن عزلات MRSA ، ثم خضعت للكشف عن جينات الضراوة (pvl و eta) ، ومورثة مقاومة المضادات الحيوية (cfr) ، وتحديد فئة Integron 1 ، و فحص تكوين الأغشية الحيوية ، و حساب فهارس MDR و MAR.

النتائج: بلغ معدل عزل S. aureus من العينات السريرية في دجاج اللحم 37.7٪. و أظهر اختبار الحساسية للمضادات الحيوية أن 85٪ من عزلات المكورات العنقودية الذهبية كانت مقاومة لواحد أو أكثر من المضادات الحيوية المختبرة. لذلك ، تم تقييم جميع العزلات الـ 53 لوجود جينات mecA و mecC بواسطة PCR. شوهد منتج PCR النوعي للجين mecA في 7 عزلات (35٪) واعتبرت من MRSA. من بين جميع عزلات بكتريا S. aureus ، كان هناك عزلتان موجبة لجين eta ، و 13 (65٪) عزلة تحتوي على مجموعة Integron class 1 ، في حين أظهر اختبار تكوين الأغشية الحيوية أن 7 (35٪) كانت موجبة لانتاج الأغشية الحيوية وثلاثة منها كانت كثيفة الانتاج. علاوة على ذلك, نتائج الدراسة اظهرت ان 13 (65٪) من العزلات كانت مقاومة لثلاث مضادات حيوية أو أكثر واعتبرت من سلالات MDR. بينما لم يتم الكشف عن جين pvl و cfr و mecC بين عزلات S. aureus.

الخلاصة: وجدنا أن المكورات العنقودية الذهبية تشكل تهديدًا حقيقيًا في صناعة الدواجن ، وهذا ينعكس لاحقًا على الصحة العامة نظرًا لاكتساب هذه البكتيريا للجينات المقاومة للمضادات الحيوية ، وأظهرت النتائج أن نسبة عالية من العزلات كانت من سلالات MDR منهم كانوا مقاومين لجميع المضادات الحيوية المستخدمة في هذه الدراسة. بالإضافة إلى وجود عزلتين من MRSA تحملان جين eta ، فهذا يشير إلى أنها من أصل بشري.

الكلمات المفتاحية: MRSA, S. aureus, mecA, mecC, pvl, eta, cfr genes, SCCmec, MDR, MAR.

Inroduction:

Staphylococcus aureus (S. aureus) is a commensal and pathogenic bacterial species for both humans and animals, that colonized 20-30% of the human population globally, and is a usual cause of infections in both the hospital and community (1). it's a dangerous gram-positive bacterial pathogen which destroys the leucocytes and evades the host's immune system and therefore causing a serious disease (2). S.aureus can cause diseases for animals and humans that ranging from osteomyelitis, meningitis, skin and soft tissue infection (SSTIs), brain abscesses, endocarditis, pneumonia to bacteremia (3). Also, besides to Escherichia coli and Proteus mirabilis, S. aureus can cause bumblefoot (4), arthritis (5), synovitis, cellulitis (6), swollen head syndrome (7), and omphalitis (8) in poultry. The pathogenicity of S. aureus was due to its ability to produce a wide range of proteins (exotoxins) that help in colonization, adhesion, and invasion of the animals and humans tissue, these

proteins classified into different types according to their mechanism of action and all of them were encoded in the chromosome, plasmid and could be some times in the transposons of this bacteria, however, this genes could be gained or lost according to the origin of strains, the host that will infect, and many other factors like transmission capacity, for example, the most common and dangerous exotoxins were the Enterotoxins that causes food poisoning, the toxic shock toxin (TSST), Exfoliative toxin (ET), and the significant Panton-Valentine Leukocidin (PVL) that causes leukocyte dissolution and therefore tissue necrosis (9). the gene-encoding PVL toxin has been found in methicillin-resistance staphylococcus aureus (MRSA) especially that originated from the community (CA-MRSA) (10). For many years the drug of choice for fighting the *S. aureus* was beta-lactam antibiotics, but in 1960 the resistance of methicillin among S. aureus isolates emerged, and these specific strains was named as MRSA (11). Most of MRSA strains are supplied by modified membrane associated penicillin binding protein (PBP2α) that expressed heterogeneously and determined by the action of mecA gene, mecA gene was found on large mobile genetic element (MGE) designated as the staphylococcus cassette chromosome (SCC), PBP2α showed low affinity for β-lactam antibiotic classes that allowed S. aureus bacteria to resist these drugs.(12,13). It was originated from different sources and environments, hospital-associated MRSA (HA-MRSA), and the community-associated MRSA (CA-MRSA) considered the primary origin of infections in humans (14). In fact, there are other strains, a dangerous threat for both humans and animals, as it has shown the possibility of zoonotic transmission, named livestock-associated MRSA (LA-MRSA) Which was found in livestock animals such as, cattle, ducks, poultry, rats, and pigs (15,16). Besides the resistance to β-lactam drugs, S. aureus has shown unique gene that code for multidrug resistance called (cfr) gene that encodes for many classes of chemically unrelated antibiotics, the most important was phenicols, streptogramin A, pleuromutilins, lincosamides, and oxazolidinones (17), These characteristics make these bacteria one of the most important hazards threatening the humans and animals alike (18). Moreover, both coagulase-positive and negative staphylococci may acquire battery of genes called integrons that can carry single or multiple gene cassettes coded for resistance to different antibiotics (19–21), The co-existence of integron and SCC*mec* in *Staphylococci* increase the risks of horizontal transfer of resistance genes (22), this problem did not stop there, The ability of S.aureus to produce biofilms increased the resistance to antibiotics and make the treatment process difficult (23,24).

Materials and methods:

Study design and Specimens collection

A cross-sectional study was performed to collect A total of 53 samples of clinical cases from broilers chickens, during the period from August 2019 to February 2020, the samples collected from different locations in Karbala and Al-Najaf cities. The samples were collected using a sterile swab from the body fluid, swabs from unabsorbed yolk sac of omphalitis infected chicks, head pus, foot bad pus, synovial fluid swabs. The sterile swab was immersed in a brain heart infusion agar tube and transferred to the

laboratory within three days or less, and cultured on blood agar and mannitol salt agar directly. The *S. aureus* isolation, detection, and classification were according to the Bergey's manual guidelines, depending on the morphological examination on the culture medium and microscope, and biochemical tests. All sample were inoculated for 24 hours at 35°C-37°C on blood agar (BA) and mannitol salt agar (MSA), colonies on blood agar were shown a clear zone of β-hemolysis, however, on MSA the colonies show the ability of mannitol fermentation and change the color of media from pink to yellow. The microscopic observation was conducted after the isolates were stained using a gram staining procedure, using AmScope40X-2500Microscope with LCD Touchpad Screen to determining the shape, color, and arrangement of the examined isolates. Then, all suspected colonies were subjected to biochemical tests (catalase, oxidase, and slide coagulase) for identification of S. aureus isolates

Susceptibility test for antimicrobials using disk diffusion (DD) method

Few colonies of the fresh isolate were selected from MSA medium and suspended with BHI broth medium to make direct colony suspension and compared visually with McFarland standard 0.5%. A sterile cotton swab was inserted into the direct suspension and dried well on the tube's inner wall, then the Muller-Hinton agar plate was inoculated using the streaking method across the whole agar surface more than three times. The discs were placed using a disc dispenser, spread over equal distances between each disc (28 mm distance from center to center) on the agar plate with a size of 90 mm. Then, incubated in an inverted position at 35°C. The calculation of inhibition zone diameter was after incubation for 18 hours, while oxacillin disks needed 24 hours of incubation before being identified as susceptible.

Definition of multidrug resistance (MDR) and index calculation of multiple antibiotics resistance MAR

MDR of *S. aureus* was described as having acquired resistance to at least one antimicrobial agent among three or more categories (25). The (MAR) index calculation was as the following relation:

$$\text{MAR} = \frac{\text{Number of antibiotics resisted}}{\text{Number of antibiotics tested}} \quad (26)$$

Table (2): antibiotic discs used in this study (MAST/USA).

Antibiotic class	Antibiotic name and content	code	Inhibition zone diameter (mm)		
			S	**I**	**R**
β-lactams	Penicillin G (10U)	PG	≥29	-	≤28
	Oxacillin (1 µg)	Ox	≥13	11-12	≤10
Aminoglycosides	Gentamicin (10 µg)	GM	≥15	13-14	≤12
macrolides	Erythromycin (15 µg)	E	≥23	14-22	≤13
Tetracyclines	Tetracycline (30 µg)	T	≥19	15-18	≤14
Fluoroquinolones	Ciprofloxacin (5 µg)	CIP	≥21	16-20	≤15
Lincosamides	Clindamycin (2 µg)	CD	≥21	15-20	≤14
Inhibitors of Folate pathway	Trimethoprim-sulfonamide (1.25/23.75 µg)	TS	≥16	11-15	≤10
Phenicols	Chloramphenicol (30 µg)	C	≥18	13-17	≤12
Ansamycins	rifampin5 µg	RP	≥20	17-29	≤16

Testing the Biofilm production ability

The biofilm production test was conducted with few modifications according to a method performed by Piechota *et al.*, 2018 The experiment was applied on all 20 *S.aureus* isolates, each isolate was grown on BHI agar supplemented with dextrose 0.5% at 37°C for 24 hours, after incubation, the bacterial colony was transferred to BHI broth supplied with 0.5 g dextrose to prepare bacterial suspension matched to McFarland's standard solution 0.5 % that equal to 10^8CFU/ml. 200 µm of the suspension transferred into wells of 96-well polystyrene plate and incubated without shaking at 37°C for 48 hours,

after second incubation the excessive medium was removed and washed 2-3 times with normal saline solution, the next step was a fixation that performed using an oven at 60°C for one hour, then 200 µm of crystal violate 1% was added for 5 minutes. after this time, the plate was rinsed with normal saline and dried with air for one hour. Colorant was solved in 96% ethanol and absorbency was measured by Absorbance microplate reader at 490 nm, each assay was conducted in triplicate to calculate the average results, Absorbance values were considered to be positive for biofilm formation at absorbency rate ≥0.12, weak biofilm producers at <0.2, moderate at 0.2-0.4, and strong producers at >0.4 (27).

Molecular methods

DNA of all isolates was extracted directly from colonies aged 24 hours, as instructed by the DNA extraction kit manufacturing company (Intron, Korea), and the primers used in this study was mentioned in tables 3, 4, and 5.

Table (3): primers used for the detection of MRSA isolates.

primer		Sequence 5'-3'	Size (bp)	Manufacturer company	references
mecA	F	TGCTATCCACCCTCAAACAGG	286	Integrated DNA Technologies, USA	Reference (28)
	R	AACGTTGTAACCACCCCAAGA			
mecC	F	TCAAATTGAGTTTTTCCATTATCA	1932	Integrated DNA Technologies, USA	(29)
	F	AACTTGGTTATTCAAAGATGACGA			

Table (4): primers used in virulence factors detection.

primer		Sequence 5'-3'	Size (bp)	Manufacturer company	references
eta	F	CGCTGCGGACATTCCTACATGG	676	Integrated DNA Technologies, USA	(30)
	R	TACATGCCCGCCACTTGCTTGT			
pvl	F	GCTGGACAAAACTTCTTGGAAT	83	Integrated DNA Technologies, USA	(31)
	F	GATAGGACACCAATAAATTCTGGAT			

Table (5): the primers that used in the identification of resistance factors

primers		**Sequence 5'-3'**	**Size (bp)**	**Manufacturer company**	**references**
cfr	F	TGAAGTATAAAGCAGGTTGGGAGTCA	746	Integrated DNA Technologies, USA	(32)
	R	ACCATATAATTGACCACAAGCAGC			
Integron class I	F	CAGTGGACATAAGCCTGTTC	160	Integrated DNA Technologies, USA	(33)
	R	CCCGAGGCATAGACTGTA			
Conserved sequence	F	GGCATCCAAGCAGCAAG	Variable	Scientific Research Co. Ltd	(34)
	R	AAGCAGACTTGACCTGA			

Monoplex PCR method

The monoplex PCR was achieved according to the manufacturing company of the master mix and the reaction mixture was prepared in a total volume of 25µl. All coagulase-positive sample were subjected to detection of the mecA gene mentioned in Table (3-5) to identifying the MRSA isolates, then all samples subjected to detection of eta and cfr genes for identification the existence of exfoliative toxin type A and multidrug resistance characteristics, then all identified MRSA isolates subjected to pvl and mecC genes detection. Furthermore, all *S.aureus* isolates were submitted to integron detection.

Statistical analysis

SPSS (version 21) was used to analyze the current data. Differences were obtained by applying Chi-squared test. Differences were setting as significant at 5% ($P \leq 0.05$) and 1% ($P \leq 0.01$).

Ethical approval

This study did not include the use of genetically changed organisms or biological materials and was carried out under the supervision and recommendations of the Faculty of Veterinary Medicine, University of Kufa, according to the controls approved by it. All samples that were worked on in this study were collected according to the research protocols for each type, without additional materials or manipulation.

Results and discussion

Sampling and isolation of *S. aureus*

A total of 53 of various clinical cases of poultry were collected and screened for *S. aureus* during the study. The samples were 20 (37.7%) omphalitis samples; 10 (18.8%) bumblefoot samples; 12 (22.6%) swollen head syndrome samples; and 11 (20.75%) arthritis samples, as shown in Table (6). While the distribution of *S. aureus* among these samples was diverse, bumblefoot were the most frequent sample for isolation of *S. aureus*, followed by arthritis samples, and raw milk samples (60%, and 54.54% respectively). The remaining isolates were identified in, Omphalitis 30%, swollen head syndrome 16.66%.

The clinical isolates were determined by using conventional standard biochemical tests. All the specimens cultured on blood agar medium supplemented with 5% blood for primary isolation and selected by using selective media (MSA) for confirmation of the mannitol fermentation, then subjected to gram's staining, catalase, oxidase, and further slide coagulase test. The ability to grow on MSA media, oxidase negative, and catalase-positive, they were identified as *staphylococci*. Among these *staphylococci*, 20 (37.7%) isolates show a positive result with a slide coagulase test and considered as *S. aureus* isolates.

Table (6): occurrence of *S. aureus* isolates according to the source of isolation

Samples		No.(%) of samples	No.(%) of *S. aureus* isolates	No.(%) of MRSA isolates
Poultry samples	Omphalitis	20 (37.7)	6 (30)	2 (33.3)
	Bumblefoot	10 (18.8)	6 (60)	1 (16.6)
	Arthritis	11 (20.75)	6 (54.54)	4 (66.6)
	swollen head syndrome	12 (22.64)	2 (16.66)	0 (0.0)
Total		**53 (100)**	**51 (45.13)**	**16 (31,3)**

The isolation rate of *S. aureus* among omphalitis clinical samples

Worldwide, infection of the yolk sac is a major challenge in the poultry industry, and its occurrence leads to huge economic losses in the first week of hatching and many studies are trying to solve this problem using different ways of control and treatment (35). In this study, the prevalence of *S. aureus* isolated from unabsorbed yolk sac of omphalitis infected chicks was 30% and this percentage agreed with the results shown by some previous studies such as

20% (Khalil, S. A., and Einas, 2012), 23.5% (37), and 23.3% (38). However, the present study disagrees with (39) that show a high prevalence rate of 55.9% and other studies that show a low isolation rate of *S. aureus* 0.5% (8).

The isolation rate of *S. aureus* among Bumblefoot clinical samples

Bumblefoot is a descriptive term for any inflammatory or degenerative poultry foot disease. It typically occurs with age (14-70 days), but the majority of cases occurred about 35 days (40). This can range from moderate redness or swelling to severe, deep-seated abscesses, and osteocytes changes (41). The prevalence of *S. aureus* isolates in the bumblefoot samples was 60% and this result is consistent with the results of a study conducted in Sulaymaniyah (4), which showed that the incidence of *S. aureus* in bumblefoot samples was 57%. Even so, these results do not conform to what a study in other countries has shown, which showed that the percentage is as follows 10% and 8.33% (42,43). The high isolation rate of *S. aureus* among bumblefoot clinical samples could be due to the environmental condition of the farms. Breeding birds in humid weather on thick, old, and contaminated litter contributing establishment the bumblefoot lesion. The entrance of *S. aureus* bacteria throw the injured skin leads to swollen the footpad and formation of abscesses (44,45)

The isolation rate of *S. aureus* among arthritis clinical samples

Arthritis is one of the major challenges that poultry suffer, associated with decreased weight gain, weakness, and death because of the infected birds struggling to get feeding, Arthritis also causes great marketing, as the customer will avoid chickens with arthritis (46). six *S. aureus* isolates were isolated from eleven arthritic birds, thus the isolation rate for these bacteria was 54.54%. and this result agreed with (47), and (48) who showed that the isolation rate of *S. aureus* in the arthritic bird was 50.98% and 46.5% respectively, However, the isolation rate could increase in some circumstances and reach to 68% according to (49). While these results disagree with studies that showed significantly lower isolation rates such as 25% (50), 19% (51), and 16.7% among different clinical cases in infected birds (52). The high isolation rate of *S. aureus* among arthritis samples was not surprising, according to Adayel, S. A. (2005), the most frequents sites of *S. aureus* infections in poultry were tendons, sheathes, bones, and joints (53) this may due to staphylococcus have a high affinity for the rich-collagen tissue and growing bone surfaces (54).

The isolation rate of *S. aureus* among swollen head syndrome clinical samples

The respiratory tract-associated infection has major economic effects on worldwide poultry development. The swollen head syndrome is an upper respiratory condition and has been regarded in recent years as one of these issues (55). In the presented study, the isolation rate of *S. aureus* among swollen head syndrome that isolated from swollen sinuses and head pus was 16.66% and this result agreed with the previous studies of (56) and (57) that showed the isolation rate of *S. aureus* among swollen head syndrome samples was 12.5% and 18.2% respectively. However, disagreed with (58) who showed that the isolation rate decreased to 2.4%.

Evaluation of Antibiotic susceptibility test (AST)

Among poultry samples, there are varying degrees of resistance for each antibiotic and in the following sequence: Chloramphenicol 11(55%), tetracycline 14(70%), Oxacillin 13(65%), trimethoprim-sulfonamide 7(35%), Rifampin 8(40%), Penicillin G 16(80%), Gentamicin 8(40%), clindamycin 14(70%), Ciprofloxacin 10(50%) and erythromycin 15(75%). The resistance rate of Chloramphenicol in the presented study agreed with (59) who showed that this rate could be ranging between (34%-69%) among poultry samples, fields, and slaughterhouses, while some studies showed that the resistance rate may be raised to reach 81% like (60). And another study shows no resistance for Chloramphenicol among *S. aureus* isolated from chicken (61). However, this study result was close to Amoako (2020) result of another resistance rate such as tetracycline 61.6%, trimethoprim-sulfonamide 30%, Penicillin G 55.8%, clindamycin 43.3%, Rifampin 40.8%, and erythromycin 54.7%, and disagreed with Gentamicin resistance rate that is 8.3%. this percent changed according to the source of isolate and the antibiotic selective pressure and many other factors affecting the antibiotic resistance pattern among poultry, so in another study conducted by Bounar (2018) who showed that the resistance rate variable among laying hens and broilers, the resistance to tetracycline ranged between 44%-74%, while in trimethoprim-sulfonamide was 21%-28%, in Penicillin G was 79%-93%, and in erythromycin was 45%-55%. While The resistance rate of oxacillin among poultry samples in this study was 50% and this percent agreed with (62) who showed that the rate of resistance among broiler and layer hens was 53% and 57% respectively. Moreover, the resistance rate of Ciprofloxacin in this study agreed with (63) who showed that the rate of resistance was 33.9%, and disagreed with the resistance rate of Gentamicin that is 19%. In this context, the rate of resistance to Gentamicin in this study was 30.77% and this percent was close to (64) result who showed that the rate was 42.3% among different samples from broiler chickens.

Multiple antibiotic resistance (MAR) indexes

The antibiotic resistance test revealed that 75% of *S. aureus* isolates tested in this study were found to have had MAR index of 0.2 and above, and 60% have had an index of 0.3 and above. According to antecedent studies, the MAR index above 0.3 suggests that bacteria had already emerged in an area in which antibiotics were routinely used (26). this study result of MAR indexes considered low comparing to several studies of the same type of samples (65,66). The variation of results between studies indicating that these different isolates emerged from a high-risk contaminated environment where antibiotics are frequently used for treatment, prevention, and growth promoters (67).

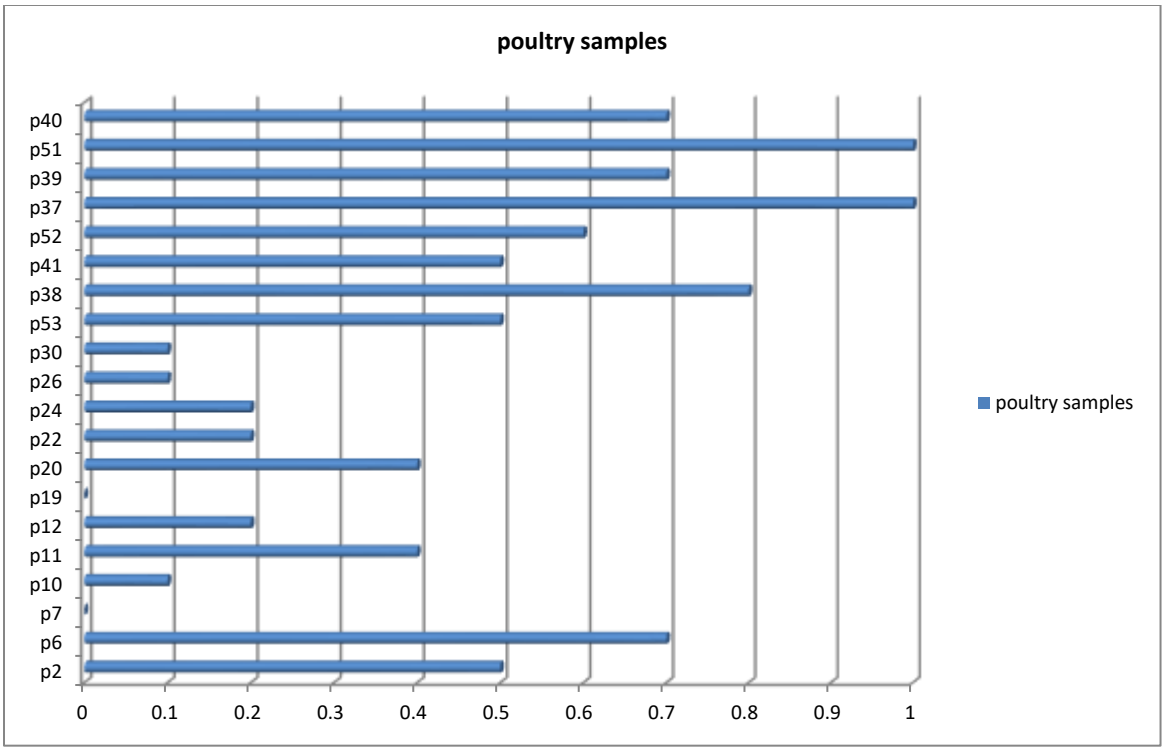

Figure 1: MAR index of S. aureus isolated from poultry clinical samples.

Multiple drug resistance (MDR) profiles

Various concepts have been used to describe the multidrug resistance (MDR), because of the different nature of tests and guidelines followed. Therefore, to avoid ambiguity, this study used a resistance pattern according to (25) that define the MDR term as the resistance of the microorganism to one of three classes of antibiotics. Few studies conducted to explain the MDR patterns in Iraq especially among animals, the present study revealed that the 12 (60%) isolates were resistant to three or more antibiotic classes. 2 (10%) isolates resist to four antibiotics classes; 3 (15%) isolates were resisted to five antibiotics among nine classes; 1 (5%) isolates were resistant to six antibiotic classes; 3 (15%) were resistant to seven antibiotic classes; 1 (5%) isolates were resisted to eight antibiotic classes; and 2 (10%) resisted to all antibiotics used in this study, while there are no isolates were resisted for three antibiotics. A study conducted by Liu *et al* (2018) was shown a pattern of multidrug resistance among *S. aureus* isolated from poultry samples as follows: 4.2% of isolates were resistant for 3 antibiotics, 3.5% for 4 antibiotics, 6.29% for 5 antibiotics, 11.89% for 6 and 7 antibiotics classes, 23% for 8 antibiotics, and 13.2% of isolates were resistant for 9 antibiotic classes (68). Differences in MDR patterns due to many factors including contamination, the birds' clinical condition, and repeated unsupervised use of antibiotics.

Prevalence of virulence and antibiotic resistance genes

All 20 *S. aureus* isolates were subjected to detection of the multidrug-resistance gene (cfr) using a specific PCR product of 746 bp, the examination

came with a negative result, as all samples are free of this gene because this gene found in coagulase-negative in high prevalence rate especially among animals like cattle, pigs, and poultry (69). furthermore, a low prevalence rate of the cfr gene was shown by (70) among *S. aureus* isolated from different animal samples. As well as all isolates submitted to the detection of exfoliative toxin gene (eta) using a product of 676 bp and there are 2 (10%) isolates were positive for this gene, demonstrated in (Figure 2). The prevalence rate in the present study was higher than (71) who found one MSSA isolate carrying this gene among 59 isolates in percent of 1.7 in poultry samples. Moreover, (72) did not find any isolate carrying this gene in different poultry samples. the existence of exfoliative toxin genes was reported in many animal species such as canine, ovine, bovine, and poultry and the role of this toxin in *S. aureus* pathogenicity is clear as it serine proteases that cleave the desmoglein-1 protein which play important role in cell-to-cell junction, that explains the epidermis exfoliation of chicks that injected with this toxin (73,74). Nevertheless, all MRSA isolates were subjected to detection of the pvl gene using a specific PCR product of 83 bp and the result was negative for all 16 isolates. The Integron analysis in the current study performed on all *S. aureus* isolates using two specific PCR products, the first one was intI1 integrase that coding to Integron class I, and the second was 3CS,5CS that coding for gene cassettes integrated into the variable region of Integron class I. The result revealed that 15 (75%) of the samples were positive for intI1 and negative for the conserved sequence (3CS,5CS) (Figure 2), this result higher than Xu *et al.*, 2007 who reported that 53% of his isolates harboring the intI1(19), and According to previous studies, the conserved sequence (3CS,5CS) could be gained or lost because it is located within the variable region of integrons (75,76).

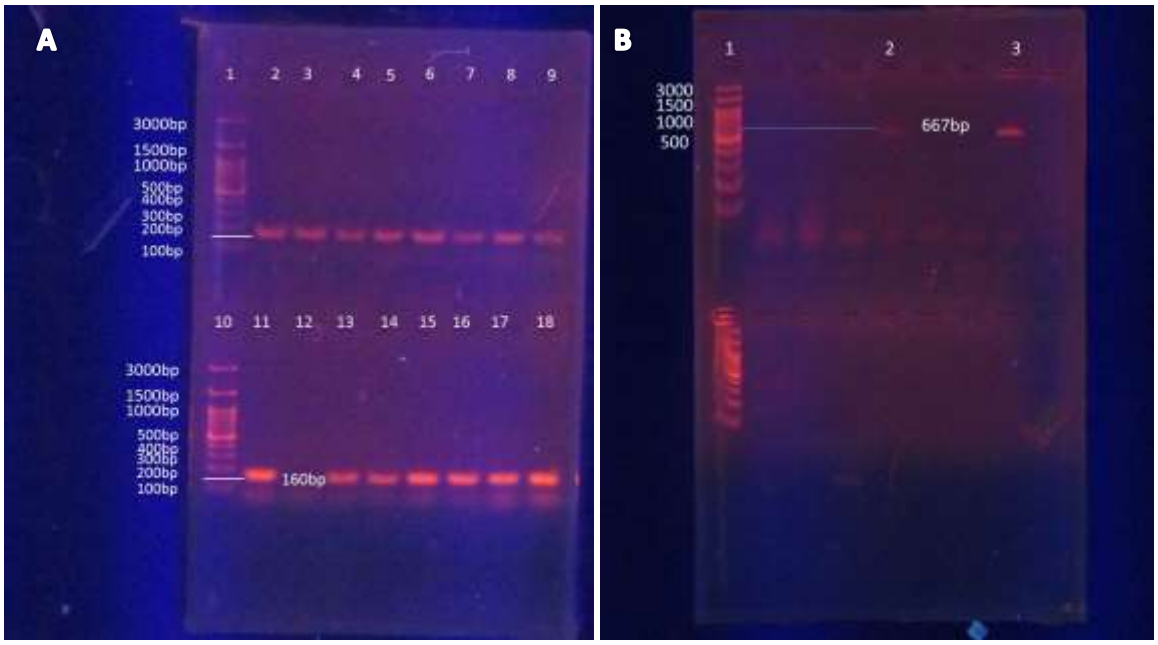

Figure 2: (A) gel electrophoresis for intI1 PCR product of 160bp. Lane 1 and 10 were DNA markers (100bp). The lane 2-9, 11, and 13-18 were positive intI1 products, lane 12 were control negative. (B) gel electrophoresis for eta PCR product of 667 bp. Lane 1 and were a DNA marker (100bp), The lane 2 and 3 were positive eta product.

Biofilm ability formation

All 20 isolates were subjected to biofilm formation test using a modified method of (27). The result of this test revealed that 7 (35%) isolates were positive biofilm producers and 3 (42.8%) of them were strong producers. While 1 (14.2%) isolates considered as a moderate producer, and 3 (42.8%) were weak biofilm producers. This study result showed a low rate of strong biofilm producers among poultry samples 42.8% comparing Ou *et al* 2020 how to show that the strong biofilm producers among *S. aureus* isolated from poultry was 64.8% were the moderate and weak producers was 20% and 15.2% respectively (77).

Identification of methicillin resistance *S. aureus*

The antibiotic susceptibility test revealed that 13 of *S. aureus* isolated from poultry samples were resistant to oxacillin. So, all 20 isolates were assessed for the presence of mecA and mecC genes by PCR. The mecA gene-specific PCR product of 268 bp was seen in 7 isolates and were considered to be MRSA. Therefore, the prevalence rate of MRSA isolates was 35%. The remaining isolates were mecA-negative (MSSA). The discrepancy in the results of antibiotic susceptibility and molecular test can be explained by the fact that bacteria may use other methods to combat methicillin and its derivatives without having to possess a mecA gene that responsible of β-lactam resistance, it can be due to many reasons, including hyperproduction of β-lactamase enzyme among mecA-negative MRSA strains

(78). Moreover, Ba and colleagues reported that there is a specific alternation in different amino acids among the proteins of protein binding cascade (PBP type 1, 2, and 3) and these features were among MRSA strains that lack the mecA gene (79). Furthermore, Banerjee reported that there are specific mecA-negative MRSA strains have expressed specific mutation in different amino acids among the protein of PBP4 that may help the bacteria in methicillin resistance (80). In 2003, Yoshida reported that the loss of a mecA gene among MRSA strains can be compensated for by acquiring a wall three times thicker than normal (81). These findings demonstrate that there are other mechanisms for resistance to methicillin and its derivatives, and the molecular methods alone are not sufficient for the definitive characterization of MRSA isolates. From the time when the first detection of MRSA in 1961 (11), MRSA has appeared world infectious problems and it is responsible for a wide range of diseases, from simple lesions to severe life-threatening diseases (82). Since MRSA strains carrying elements that encoding the resistance to all β-lactam antibiotics as well as the treatment options are limited significantly (83).

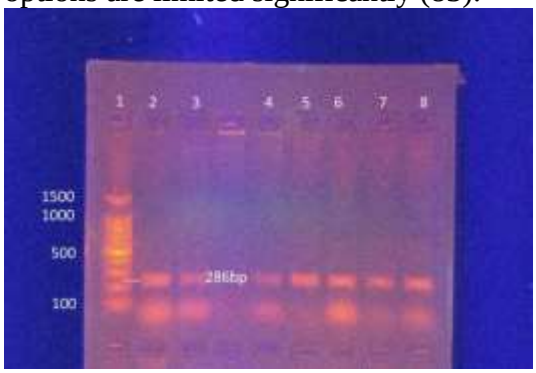

Figure 3: Gel electrophoresis of the mecA gene among *S. aureus* isolated from poultry samples. Lane1, DNA Marker (100bp), lane 2-8, mecA positive.

In recent years, MRSA has become a particularly significant problem in many regions on the world because of its ability to getting resistance to antimicrobial drugs (84), thus, the rapid recognition of MRSA was necessary for prompting effective treatment and preventing the dissemination of infection (85). Furthermore, the prevalence of MRSA differs considerably from one region to another and among farms in the same district, the data on the prevalence of MRSA in Iraq is limited, few studies were reported different prevalence rate in chickens, and slaughterhouses as follows: 61.1% and 53.8% (86,87), and 66.6%, 20.3%, and 27.3% among chickens and attached stations for slaughterhouses and poultry fields (42,88,89). Meanwhile, the prevalenve rate of MRSA in other countries was as follow 44% among chickens carcasses in Egypt (90), 9.5% among poultry meat in Jordan (91), in Algeria the prevalence of MRSA among broilers was 20%-50% according to (62,92) while in layer chickens in Egypt the prevalence was 60% (93).

In conclusion, the current study revealed that *S. aureus* constituted a real threat in the poultry industry, reflecting a public health problem due to the large acquisition of antibiotic resistance genes by these bacteria, the results indicated that a high percentage of isolates were MDR-type, and two of them were resistant to all antibiotics used in this study. In addition to the presence of two MRSA isolates carrying the eta gene, this indicates that they are of human origin. Finally, the transmission of these bacteria from humans to animals and vice versa may constitute a real

challenge in controlling these bacteria later.

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