

Detection of antibiotic resistance genes in *Staphylococcus aureus* bacteria isolated from arthritis in broiler chickens, Aleppo countryside, Syria

***S. aureus* resistance in broiler arthritis**

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Abstract:

Introduction: The danger of *Staphylococcus aureus* as a leading human and animal pathogen lies in its antimicrobial resistance, which renders standard antibiotics ineffective and severely restricts treatment possibilities.

Material and Methods: This study was conducted in the period between January 2026 and February 2026, as samples were collected from 55 farms suffering from cases of lameness in chickens. *S. aureus* bacteria were isolated using selective media, then antibiotic susceptibility testing was performed, and some antibiotic resistance genes

were detected using PCR technique, including *mecA*, *aphD*, *tet-K*, *tet-M*, *erm-C* and *vat B*.

Results: According to the results, pathogenic *S. aureus* accounted for 45.45% of the examined broiler joint lesion cases. The results also showed that all *S. aureus* isolates belonged to the multidrug-resistant (MDR) category, as they were highly resistant to penicillin G at 100%, followed by streptomycin at 96%, while the highest sensitivity rate was to tilmicosin at 64%, followed by oxacillin at 56%. The methicillin resistance gene *mecA* was detected in 24% of isolates, the aminoglycoside resistance gene was found in 100% of isolates, 80% of isolates carried the tetracycline resistance gene *tet-K*, while the tetracycline resistance gene *tet-M* was found in 48% of isolates, whereas the macrolide resistance gene *erm-C* was detected in 72% of isolates, and the streptogramin resistance gene *vat B* was not detected in any of the studied isolates.

Conclusion: We conclude from this study antibiotic-resistant *S. aureus* bacteria, which portends the transmission of this resistance to other bacteria that infect humans, threatening public health.

Keywords: antibiotics, arthritis, broilers, lameness, resistance genes, *Staphylococcus aureus*.

1. Introduction

S. aureus is a significant opportunistic pathogen affecting both human and animal health, causing various infectious conditions and foodborne illnesses [1]. Globally, it ranks third among foodborne disease agents in terms of both public health impact and economic burden, Although this bacteria commonly colonizes on the skin and nasal passages in animals without causing symptoms, it has the potential to induce skin and soft tissue infections under certain circumstances [2].

51 *S. aureus* is a Gram-positive cocci, it classified under the genus *Staphylococcus* within
52 the Micrococcaceae family. The term 'staphylococci' derives from their unique division
53 pattern, where cells undergo multiple divisions in different planes, resulting in
54 characteristic grape-like clusters of spherical organisms [3].

55 *S. aureus*-associated disease syndromes impose substantial financial strain on the global
56 poultry sector and threaten economic stability. Specifically, this pathogen leads to
57 considerable economic damage through lameness, increased death rates, reduced weight
58 gain, diminished egg output, and carcass condemnation at processing facilities [4].

59 Lameness severity varies from slight gait abnormalities to complete immobility, where
60 birds rely on their wings for balance and movement. Severely affected individuals
61 become agitated and struggle to reach feed and water. Structural defects and lameness-
62 related factors may also arise from production-related processes, leading to profound
63 and permanent consequences in commercial poultry operations [4].

64 The cause of infection-based lameness in poultry is not fully understood. However,
65 several studies have indicated that microorganisms enter the bloodstream of birds,
66 mainly through the gastrointestinal and respiratory tracts, and the pathogens reach the
67 joints [5].

68 In birds, arthritis followed by lameness typically develops after blood infection
69 (bacteriemia) or localized joint invasion by bacterial pathogens such as *S. aureus* [4].

70 The primary targets for staphylococcal lesions include bones, tendons, and joints,
71 whereas sites like the skin, sternal bursa, yolk sac, heart, eyelids, and testicles are less
72 frequently affected. Osteomyelitis and arthritic changes commonly induce lameness in
73 commercial poultry, particularly among breeder and ornamental birds [5].

Zoonotic transmission of livestock- associated methicillin-susceptible *S.aureus* (LA-MSSA) and methicillin-resistant *S. aureus* (LA-MRSA) from food-producing animals to humans has been reported in many studies worldwide [6].

Various antibiotic agents are widely used as growth promoters, for prevention, and for treatment of bacterial infections in broiler chicken farms due to their intensive nature and high demand. Misuse of antibiotics can lead to antibiotic resistance in microorganisms, including *S. aureus*. Additionally, *S. aureus* has developed multiple resistance genes and thus has become more virulent [7]. The need for alternative solutions to antibiotic use has increased, and the major problem lies in the transfer of antibiotic resistance genes either through vertical transfer or through horizontal transfer to other bacteria, heralding the approach of the post-antibiotic era, where bacteria will not respond to any type of antibiotic [8]. Therefore, we conducted this study as a contribution to determining the resistance rate of *S. aureus* isolates from arthritis to antibiotics and detecting the genes encoding some of these antibiotics.

2. Materials and methods

2.1. Sample collection:

55 samples were collected to conduct the study from 55 broiler farms in the Manbij region in the countryside of Aleppo governorate in Syria . Within a period not exceeding 10 hours after the birds death, the chicken carcasses were then transferred to the laboratory , in order to perform the pathological autopsy by the veterinarians specialized in the farms. The samples were obtained after death from chickens that had died in the farm due to some disease, or were humanely euthanized in the farm by the veterinarians or trained workers due to their deteriorating health condition as a result of lameness . The sampling site was well sterilized to avoid contamination, and the

samples were taken, where the samples were in the form of swabs of the synovial fluid and the bone head in the affected joint , where both the hip joint and the hock joint were targeted , and the swabs were placed in peptone water and incubated at 37°C for 24 hours.

The bone lesions in the proximal end of the femur associated with the hip joint included three types: femoral head separation (FHS), transient femoral head necrosis (FHT), and femoral head necrosis (FHN), with the latter being the most common. Regarding the hock joint, tibial head necrosis (THN) was observed in the proximal end of the tibia, which is the characteristic lesion of this joint.

2.2.Bacterial isolation:

The collected swabs were streaked onto Chapman Stone (mannitol salt agar) culture medium. Following incubation, colonies capable of fermenting mannitol, which appeared with a characteristic golden-yellow pigmentation, were selected for further analysis. Gram staining was conducted for microscopic assessment of cellular morphology, followed by biochemical profiling using the HiStaph™ KB004 identification kit. The coagulase test was also carried out to confirm species identity. Ultimately, pure cultures were maintained on nutrient agar slopes for subsequent experiments.

2.3.Antibiotic susceptibility testing:

The test was performed using the disc diffusion method in Mueller-Hinton Agar specific for antibiotic susceptibility testing at a concentration of 0.5 McFarland, and using single antibiotic discs (Antibiotic Unidisc), Which included oxacillin, ampicillin, cloxacillin, ceftiofur, ciprofloxacin, enrofloxacin, erythromycin, streptomycin,

gentamicin, neomycin, oxytetracycline, doxycycline, chlortetracycline, penicillin G, amoxicillin, sulfamethoxazole-trimethoprim, tylosin, and tilmicosin.

The medium was incubated at 37°C for 24 hours, and the inhibition zone diameter for each antibiotic was measured using a graduated ruler [9].

2.4. Detection of antibiotic resistance genes:

2.4.1. DNA extraction:

A single colony from nutrient agar medium was transferred to 50 µl of distilled water, and the tube was placed in a water bath at 100°C for 10 minutes, then rapidly cooled and centrifuged at 10,000 G, then the supernatant was taken and used as a DNA template [10].

2.4.2. Multiplex PCR reaction:

Some antibiotic resistance genes were detected along with detection of (16srDNA) as an internal control using primers shown in Table 1, and the reaction mixture was prepared in a final volume of 25 µl (3 µl DNA template, 0.5 µl forward primer and also reverse primer for each gene, 2.5 µl PCR buffer, 1 µl MgCl₂, 0.5 µl dNTP, 0.5 µl Taq enzyme, water up to 25 µl), and the temperature and cycling program which is reported previously in [11] was followed, where initial denaturation at 94°C for 1 minute for one cycle, amplification for 30 cycles (secondary denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute), and final extension at 72°C for 10 minutes.

The PCR products were detected by electrophoresis on 2% agarose gel stained with ethidium bromide.

Table 1. Primers used for detection of antibiotic resistance genes [11].

Gene	Symbol	Primer sequence 5'-3'	Product size (bp)
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Methicillin resistance gene	mecA	F	AAA ATC GAT GGT AAA GGT TGG C	532
		R	AGT TCT GCA GTA CCG GAT TTG C	
Aminoglycoside resistance gene	aphD	F	TAA TCC AAG AGC AAT AAG GGC	227
		R	GCC ACA CTA TCA TAA CCA CTA	
Tetracycline resistance gene	tet- K	F	GTA GCG ACA ATA GGT AAT AGT	360
		R	GTA GTG ACA ATA AAC CTC CTA	
Tetracycline resistance gene	tet-M	F	AGT GGA GCG ATT ACA GAA	158
		R	CAT ATG TCC TGG CGT GTC TA	
Macrolide resistance gene	erm-C	F	AAT CGT CAA TTC CTG CAT GT	299
		R	TAA TCG TGG AAT ACG GGT TTG	
Streptogramins resistance gene	Vat-B	F	GCT GCG AAT TCA GTT GTT ACA	136
		R	CTG ACC AAT CCC ACC ATT TTA	
Internal control	16S	F	CAG CTC GTG TCG TGA GAT GT	420
	rDNA	R	AAT CAT TTG TCC CAC CTT CG	

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145 2.5.Statistical analysis:

146 Microsoft Excel 2019 and GraphPad Prism (version 8.2.1) software were used for data
147 processing and charting.

148 3. Results

149 3.1.Isolation of *Staphylococcus aureus*:

150 A total of 22 isolates (88%) of *S. aureus* were obtained on culture media using
151 biochemical tests from these lesions, and 3 isolates (12%) were isolated from chickens
152 suffering from lameness but without obvious macroscopic pathological lesions, as
153 shown in Table 2.

154 **Table 2.** Number of lesions and number of *Staphylococcus aureus* isolates from cases
155 of femoral and tibiotarsal arthritis

Condition	Number of lesions / 55 cases	Percentage of lesions (%)	Number of <i>S. aureus</i> isolates	Percentage of <i>S. aureus</i> isolates(%)
FHS	4	7.27	2	8
FHT	5	9.09	2	8
FHN	30	54.55	13	52
THN	22	40	5	20
No visible gross lesions	6	9.09	3	12

3.2. Antibiotic susceptibility of *S. aureus*:

The results of this study showed widespread antibiotic resistance to *S. aureus* bacteria, with the highest resistance rate being to penicillin G at 100%, followed by streptomycin at 96%, while the highest sensitivity rate was to tilmicosin at 64%, followed by oxacillin at 56%. Table (3). Upon studying the resistance of *S. aureus* isolates according to antibiotic categories, it was found that 100% of the isolates belonged to the multidrug-resistant (MDR) group, Table (4).

Table 3. Antibiotic susceptibility pattern of *S. aureus* isolates recovered from joint infections in broilers.

Antibiotics	Sensitive	Intermediate	Resistance
oxacillin	56%	20%	24%
Ampicillin	36%	24%	40%
Cloxacillin	40%	28%	32%
penicillin G	0%	0%	100%
Amoxicillin	4%	4%	92%
Ceftiofur	48%	24%	28%
Ciprofloxacin	52%	36%	12%
Enrofloxacin	48%	28%	24%
Streptomycin	4%	0%	96%
Gentamicin	8%	8%	84%
Neomycin	12%	4%	84%
Oxytetracycline	8%	8%	84%
Doxycycline	24%	12%	64%
Chlortetracycline	32%	16%	52%
erythromycin	12%	12%	76%
Tylosin	36%	28%	36%
Tilmicosin	64%	16%	20%
sulfamethoxazole-trimethoprim	52%	20%	28%

Table 4. Distribution of antibiotic resistance class patterns of *S. aureus* isolates

Antimicrobial resistance categories*	MDR Number (%)
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β-lactamRs, AGRs, TETRs, QRs, FORs, MAC-R	13	52%
β-lactamRs, AGRs, TETRs, QRs, MAC-R	4	16%
β-lactamRs, AGRs, TETRs, QRs, FORs	3	12%
β-lactamRs, AGRs, TETRs, MAC-R	2	8%
β-lactamRs, AGRs, TETRs, QRs	1	4%
β-lactamRs, AGRs, QRs, FORs, MAC-R	1	4%
β-lactamRs, TETRs, QRs, FORs, MAC-R	1	4%
Total	25	100

*An isolate that showed resistance to one antimicrobial agent within a group was considered resistant to that group :β-lactamRs: Beta-lactam resistance, AGRs: Aminoglycoside resistance, TETRs: Tetracycline resistance, QRs: Quinolone resistance, FORs: Folate antagonist resistance,MAC-R: Macrolide resistance

3.3.Detection of antibiotic resistance genes in *S. aureus*:

Results of detecting antibiotic resistance genes in *S. aureus* bacteria isolated from joint infections in broilers showed that the methicillin resistance gene *mecA* was detected in 24% of the isolates, while the aminoglycoside resistance gene was found in 100% of the isolates. It was also found that 80% of the isolates carried the tetracycline resistance gene *tet-K*, whereas the tetracycline resistance gene *tet-M* was found in 48% of the isolates, while the macrolide resistance gene *erm-C* was detected in 72% of the isolates, and the streptogramins resistance gene *vat B* was not detected in any of the studied isolates.

All isolates were phenotypically resistant to penicillin (100%), but the *mecA* gene was found in only 24% of isolates. This indicates the presence of resistance mechanisms other than *mecA* for penicillins. Meanwhile, all isolates that showed phenotypic resistance to aminoglycosides (such as streptomycin and gentamicin) carried the *aphD* gene, indicating concordance between genotypic and phenotypic patterns. Among isolates phenotypically resistant to tetracyclines (oxytetracycline, doxycycline,

chlortetracycline), 80% carried one or both genes, while 20% of isolates were phenotypically resistant but did not carry either gene. Also, among isolates phenotypically resistant to erythromycin and tylosin, 72% carried the erm-C gene, while the remaining 28% of isolates were phenotypically resistant without having this gene. as shown in Table (5) and Figure (1) shows the electrophoresis results of PCR products.

Table 5. Prevalence of antibiotic resistance genes in *Staphylococcus aureus* isolates

N O	Antibiotics to which isolates were resistant	Detected resistance genes						
		rrnA	16S	mecA	aphD	tet-K	tet-M	erm-C
1	OXA, AMP, CLO, PEN, AMO, CEF, STR, GEN, NEO, OXY, DOX, ERY, TYL	+	+	+	+	+	+	+
2	AMP, PEN, AMO, STR, GEN, NEO, OXY, ENR, TYL	+	-	+	+	-	+	+
3	PEN, AMO, CLO, CIP, ENR, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL, TIL, SXT	+	-	+	+	+	+	+
4	PEN, AMO, GEN, OXY, ENR	+	-	+	+	+	-	-
5	AMP, PEN, AMO, ENR, STR, GEN, OXY, DOX, ERY, TYL	+	-	+	+	+	+	+
6	PEN, AMO, STR, NEO, DOX, CHL, ERY, TIL	+	-	+	+	+	+	+
7	OXA, AMP, CLO, PEN, AMO, CEF, CIP, ENR, STR, GEN, NEO, OXY, CHL, ERY, TYL	+	+	+	+	-	-	-
8	PEN, AMO, CEF, STR, GEN, NEO, OXY, CHL, ERY, TYL	+	-	+	+	-	+	+
9	PEN, AMO, STR, GEN, NEO, CHL, ERY	+	-	+	+	+	-	-
10	PEN, AMO, STR, GEN, NEO, OXY, ERY	+	-	+	-	-	+	+
11	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL	+	-	+	+	-	+	+
12	OXA, AMP, CLO, PEN, AMO, CEF, CIP, ENR, STR, NEO, OXY, CHL, ERY, TYL, SXT	+	+	+	+	+	+	+
13	PEN, AMO, STR, OXY, DOX	+	-	+	-	-	-	-
14	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL	+	-	+	+	-	+	+

15	OXA, AMP, CLO, PEN, AMO, CEF, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL	+	+	+	-	-	-	-
16	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL	+	-	+	+	-	+	-
17	PEN, STR, GEN, NEO, OXY, DOX, CHL	+	-	+	+	+	-	-
18	OXA, AMP, CLO, PEN, AMO, CEF, ENR, STR, GEN, NEO, OXY, CHL, ERY, TYL, TIL	+	+	+	-	-	+	-
19	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL	+	-	+	+	+	+	-
20	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL, SXT	+	-	+	+	+	-	-
21	OXA, AMP, CLO, PEN, AMO, CEF, STR, GEN, NEO, OXY, CHL, ERY, TYL	+	+	+	-	-	+	-
22	PEN, AMO, STR, GEN, NEO, OXY	+	-	+	+	+	+	-
23	PEN, AMO, STR, GEN, NEO, OXY, DOX, ERY	+	-	+	+	-	+	-
24	PEN, AMO, STR, GEN, NEO, OXY, DOX, CHL, ERY, TYL, SXT	+	-	+	+	+	+	-
25	PEN, AMO, STR, GEN, NEO, OXY, CHL, ERY, TYL	+	-	+	+	-	+	-
Total		25	6	25	20	12	18	0
Frequency of detected genes (%)		100 %	24 %	100 %	80 %	48 %	72 %	0 %

195 OXA : Oxacillin, AMP : Ampicillin, CLO : Cloxacillin, PEN : Penicillin G, AMO :
 196 Amoxicillin, CEF : Ceftiofur, CIP : Ciprofloxacin, ENR : Enrofloxacin, STR : Streptomycin,
 197 GEN : Gentamicin, NEO : Neomycin, OXY : Oxytetracycline, DOX : Doxycycline, CHL :
 198 Chlortetracycline, ERY : Erythromycin, TYL : Tylosin, TIL : Tilmicosin, SXT =
 199 Sulfamethoxazole-Trimethoprim

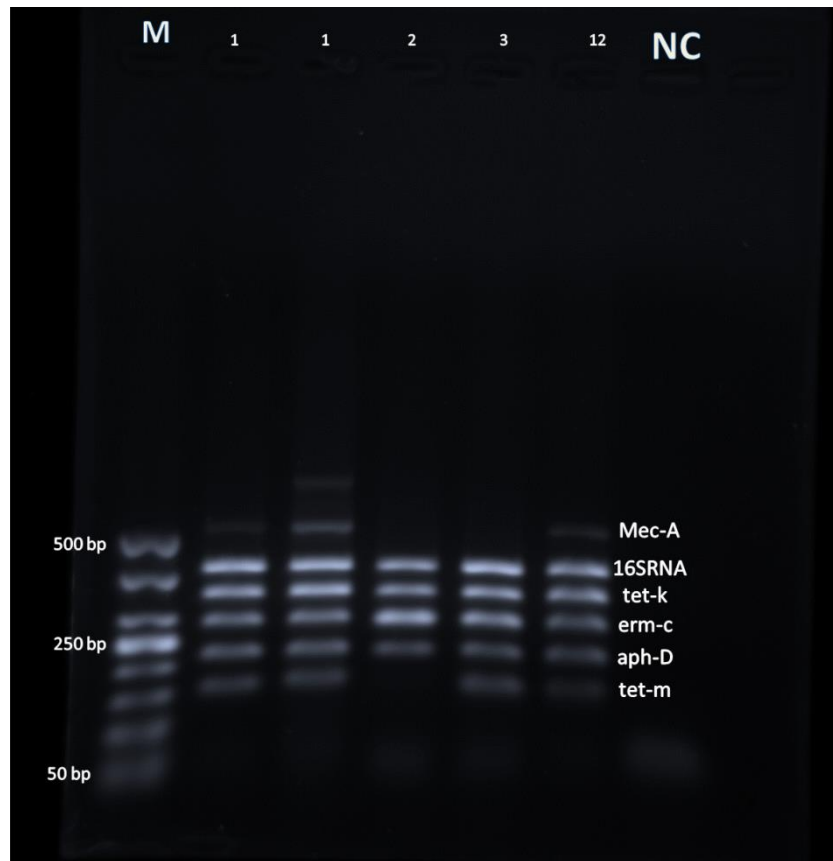


Figure 1. Agarose gel electrophoresis product of multiplex PCR, for the presence of *mec-a* (532 bp), *16sRNA* (420 bp), *tet-k* (360 bp), *erm-c* (299 bp) and *tet-m* (168 bp) genes in different *S. aureus* strains. Lane M: 50 bp ladder as a molecular size DNA marker and Lane NC: Negative control

4. Discussion

S. aureus ranks among the leading global causes of foodborne illness, frequently linked to food poisoning outbreaks. Indiscriminate veterinary antibiotic use promotes multidrug-resistant (MDR) strains, raising concerns about their transmission to humans. Poultry-derived retail products represent a major transmission route to humans, alongside occupational exposure in farms where live birds may infect workers. Human acquisition can occur through improper carcass handling, inadequate cooking of contaminated meat, or hospital-acquired spread from infected to healthy individuals [12].

Lameness-related arthritis in broilers causes considerable economic losses, as affected birds struggle to access feed and water, resulting in pronounced weight reduction or mortality. In poultry, bacterial arthritis commonly follows septicemia or localized infections, with *Staphylococcus* species being frequently implicated [13]. Staphylococcal outbreaks manifesting as arthritis and severe synovitis can emerge abruptly, presenting major health challenges [14].

In this study, out of 55 broiler samples from different farms, 25 samples were positive for pathogenic *S. aureus* (confirmed by coagulase test) at a rate of 45.45%. They were isolated from cases of hip joint and hock joint arthritis. Comparing the results of this study with previous studies, we find that it is lower than that found by [13] in Egypt, where the isolation rate of *S. aureus* from joint infections was 52.8%. A study by [15] in Pakistan reported that *S. aureus* is responsible for septic arthritis in the majority of infections in both broilers and layers, with higher prevalence in hock joints. Furthermore, layers showed a slightly lower prevalence of *S. aureus* at 64% compared to broilers at 68%, with no recorded infection of *S. aureus* causing footpad lesion swellings in layers. Meanwhile, [16] in Iraq found that the isolation rate of *S. aureus* from clinical samples of broilers was 37.7%. Epidemiological analysis conducted by [17] in Egypt revealed that MRSA-associated arthritis was detected in 15% of farms affected by lameness cases, as the *mecA* and *nuc* genes were detected in all isolates, where *S. aureus* was isolated from 60% of footpad abscesses and 65% of hock joint swellings.

Antibiotic therapy continues to be the primary control strategy; however, achieving therapeutic concentrations at localized infection sites is problematic due to inadequate drug penetration. The presence of necrotic bone lesions further complicates chronic

infection management by impeding antibiotic efficacy [14]. As a result of random and unregulated antibiotic use, resistant strains have emerged. The results of this study showed widespread antibiotic resistance, with an MDR rate of 100% of isolates obtained from joint infections in poultry. Comparing our results with regional studies reveals notable variability. While Matos et al. [24] reported susceptibility to spectinomycin, tilmicosin, and tylosin in arthritic isolates, Torky et al. [13] documented oxytetracycline and amoxicillin resistance. Almousawi and Alhatami [16] found 85% resistance to at least one antibiotic, with 65% MDR and 35% methicillin-resistant isolates carrying *mecA*. More recently, El-Sharkawy et al. [17] reported that all MRSA isolates exhibited multi-beta-lactam resistance, with ampicillin-sulbactam and vancomycin showing greatest efficacy.

Notably, most phenotypic resistance patterns in our study corresponded with the detected resistance genes. Discrepancies observed in some isolates suggest the possible involvement of alternative, undetected genetic determinants. In line with this, Hermana et al. [18] in Indonesia reported 96.2% phenotypic penicillin resistance, yet only 42.3% carried *bla*TEM, implying alternative resistance pathways. Similarly, despite 96.2% phenotypic macrolide resistance, *ermB* was found in merely 19.2% of their isolates, indicating roles for *ermA*, *ermC*, or *msrA*. Additionally, all their isolates exhibited tetracycline resistance phenotypically, whereas *tetA* was present in just 30.8%, pointing to *tetK*, *tetM*, or efflux pump involvement. Additional regional studies have documented varying resistance gene frequencies. Shaheen et al. [19] identified *mecA* and *bla*Z in all *S. aureus* isolates from chick yolk sac infections. Elsayed et al. [20] recovered 159 MRSA isolates from Egyptian broilers, all harboring both *mecA* and *femA*. Meanwhile, Faraj et al. [21] examined retail chicken meat isolates and reported the following gene

prevalences: tetA (93.8%), norA (84.4%), chlA (81.3%), blaZ (43.8%), mecA (9.4%), tetM (3.1%), and ermA (9.4%).

Discrepancies between our findings and those of earlier reports likely reflect regional differences in veterinary treatment protocols, antimicrobial selection practices, and the intensity of antibiotic use for growth promotion and disease management.

At the molecular level, antimicrobial resistance emerges from bacterial genetic alterations—either spontaneous mutations or the uptake of exogenous resistance determinants from neighboring bacteria. Such horizontal gene transfer can occur via conjugation, bacteriophage transduction, or mobile genetic elements [22].

Such resistant strains present a direct public health hazard, transferring to humans via contaminated food consumption (e.g., meat and animal-derived products) or direct contact with colonized animals. This transmission results in challenging-to-manage infections alongside elevated morbidity and mortality. Consequently, there is an urgent requirement for integrated approaches encompassing rigorous antimicrobial stewardship, novel therapeutic development, and reinforced global surveillance frameworks for antimicrobial resistance [23].

5. Conclusions:

Our findings confirm a high prevalence of multidrug-resistant *S. aureus* associated with broiler joint infections in the Manbij district (Aleppo countryside). The extensive distribution of resistance genes raises serious concerns regarding potential horizontal transfer to human-pathogenic bacteria, thereby endangering public health. Routine surveillance of antimicrobial resistance in poultry production systems is strongly recommended.

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Authors' contributions:

Haj Al Ali contributed to the acquisition of data by collecting the infected birds and performing the necropsy. A. Albaker and M. Saleh contributed to the acquisition and interpretation of bacteriological data. M. Kassouha contributed to the acquisition and interpretation of molecular data. A. Hallak contributed to the conception of the work, performed the formal data analysis, and drafted the manuscript. All authors critically revised the manuscript for important intellectual content, gave final approval for the version to be published, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest:

The authors declare no conflict of interest.

Ethical approval :

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Data availability:

All data are provided in the revised manuscript.

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