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Molecular and Bacteriological Studies on *Staphylococcus aureus* Cause Dead in Shell Eggs

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Abstract

Staphylococcus aureus is a significant pathogenic bacterium commonly implicated in the contamination of poultry products, including eggs. This study was designed to examine the molecular and bacteriological properties of *S. aureus* isolated from dead-in-shell eggs and surrounding environments (nests and hatcheries). Approximately 350 samples were aseptically collected from the internal organs of deceased embryos, egg nests, and hatcheries. *S. aureus* was isolated with a percentage of 49.8% of the total samples. The phenotypic evaluation of biofilm formation, revealed that 50 of tested isolates demonstrated biofilm-forming capabilities with different degrees. Antimicrobial susceptibility assays revealed widespread resistance among (50) representative isolates were tested, including (100%) resistance to erythromycin and rifampin, substantial resistance to chloramphenicol (88%), sulfonamides (76%), doxycycline (62%), and cefoxitin (50%), thereby classifying these isolates as MRSA. Analysis of multidrug resistance (MDR) indicated elevated resistance indices, with 22 isolates designated as extensively drug-resistant (XDR) and 28 as MDR. The MDR index values > 0.2. Molecular characterization using PCR confirmed the presence of the *16s* rRNA gene in all examined isolates. Virulence genes *eno*, *spa*, and *clf*B were identified in 100%, 71.4%, and 57.1% of isolates, respectively. The antimicrobial resistance genes *bla*Z, *mec*A, and *tet*K were identified in 85.7%, 100%, and 100% of isolates, respectively, while *van*A was present in 42.8%, and *van*B was absent. The findings underscore the considerable prevalence of highly resistant biofilm producing *S. aureus* in poultry habitats, highlighting the Important role of *S. aureus* as a causative agent of dead in shell embryos and the role of hatcheries in the spreading of the infection all over the poultry production process.

Keywords: *S. aureus*; Dead-in-shell eggs; Biofilm formation; Antimicrobial resistance; Molecular characterization

1. Introduction

A significant strategic aspect for enhancing production efficiency in the poultry business is the sanitation of viable eggs. Reducing the microbial load on eggshells might diminish the incidence of pathogenic microorganisms detrimental to embryonic development, hence enhancing hatchability and chick quality [1]. The colonization of microbiota on the eggshell initiates in the hen's oviduct, prompting enquiries on the potential adverse impact of oviductal bacteria on embryonic development. Microbiota colonization of newly placed eggshells has garnered increased attention due to its recognized link to embryonic illnesses caused by bacterial infiltration [2]. Contamination of eggshells may arise from the handler and the environment during storage and transportation [3].

Bacterial infections are among the primary causes of embryonic mortality in poultry, which include, in particular, "dead-in-shell" cases during the late stages of incubation. In the case of bacterial pathogens, *Staphylococcus aureus* is often associated because it can be introduced in the egg via eggshell invasion, or transovarian transmission causing systemic

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infection in the developing embryo. Upon entry into the egg, *S. aureus* may be able to colonize various embryonic organs (liver, yolk sac, air sac), resulting in septicemia and organ failure, in the end leading to embryo death. These infections are not only detrimental to hatchability, but also pose a serious concern to biosecurity and economics in exogenous and endogenous source poultry [2, 3].

S. aureus a gram positive aerobic bacterium found in the skin and mucous membranes of humans and animals. It can be an opportunistic pathogen whose range of infections cover from skin diseases to systemic diseases such as pneumonia, septicemia, and endocarditis. *S. aureus* also has the potential to produce biofilms and numbers of virulence factors and also has the ability to establish a resistance to the antibiotics such as methicillin resulting in MRSA. Owing to its ability to adapt and survive in different assays, this ciliate is a major concern in human and veterinary health [4, 5].

S. aureus commonly occurs in poultry farms and colonizes the skin, respiratory and gastrointestinal tracts of birds without apparent disease in many cases. It is usually non-pathogenic, unless the bird is under stress or immunocompromised in which case it may cause a variety of infections including omphalitis, yolk sac infections, septicemia, arthritis and endocarditis [5]. The pathogen is also frequently recovered from hatcheries, eggshell surfaces, and hatch residue, demonstrating its environmental survivability and involvement in both vertical and horizontal transmission. Virulence factors of *S. aureus* account for its pathogenicity and consist of exotoxins, enzyme, surface proteins and capability to establish biofilms that contribute to tissue colonization, immune escape and antimicrobial resistance. These traits render *S. aureus* an important source of mortality and decreased productivity in poultry enterprises *S. aureus* is recognized for producing numerous virulence factors, including haemolysins, leukocidins, proteases, enterotoxins, exfoliate toxins, and immune-modulatory factors [4].

Biofilm is a complex structure composed of microbial populations, featuring diverse bacterial colonies or monospecies cell types that adhere to a growing surface. It mostly consists of extracellular DNA, proteins, and polysaccharides, exhibiting significant resistance to antibiotics and physicochemical tolerance. Not all microorganisms can generate biofilm; nevertheless, certain bacteria (both Gram-negative and Gram-positive), fungus, and protests are capable of biofilm production. Many bacteria, including *E. coli, Pseudomonas, Salmonella, and S. aureus*, are capable of producing a biofilm [6].

Eid et al., [7]. examined multidrug-resistant bacterial pathogens in eggs sourced from backyard hens, with an emphasis on *S. aureus*. isolates. The research revealed that these isolates exhibited resistance to various antibiotics, including methicillin, oxacillin, and penicillin. The existence of this resistance underscores the increasing difficulty of controlling *S. aureus* infections in poultry, particularly in environments characterized by prevalent and frequently unregulated antibiotic usage.

Virulence genes encode proteins that are crucial for the successful establishment of a bacterial infection in the host organism. For instance, *S. aureus* isolates from deceased embryos have been identified to possess both *mec*A, which imparts methicillin resistance, and several virulence characteristics that enhance the bacterium's pathogenicity. This combination renders MRSA infections especially challenging to manage in chicken farms, where they can proliferate swiftly and result in considerable mortality [8, 9]. The *ica*A and *ica*D genes were identified in 58.34% and 47.22% of *S. aureus* isolates obtained from egg samples in Ludhiana, Punjab. A study evaluated the frequency, virulence traits, antibiotic resistance, and biofilm-forming capacity of *S. aureus* isolated from eggs *spa*A gene is a crucial virulence component that allows *S. aureus* to circumvent host defense responses [10].

The coexistence of *mec*A and *bla*Z in a single bacterial strain can confer resistance to a wide array of medicines, including penicillins and cephalosporins, rendering infections nearly untreatable with standard antibiotics. Moreover, the existence of virulence factors like Panton-Valentine leukocidin, a toxin that obliterates white blood cells, amplifies the pathogenicity of these resistant strains, resulting in more severe infections that are more challenging to control in poultry populations [5, 6]. *S. aureus* possesses the *bla*Z gene, which encodes a β-lactamase enzyme that inactivates β-lactam antibiotics, hence complicating treatment alternatives [9]. Therefore this study was conducted to study the molecular and bacteriological of the *S. aureus* cause dead in shell eggs.

2. Material and methods

2.1. Ethical approval

This study was approved by the ethical committee of Faculty of Veterinary Medicine, Suez Canal University with the number of 2019069.

2.2. Sample collection

A total of 350 samples were collected under strictly aseptic conditions. Samples were collected from deceased in-shell embryos (air sacs, yolk sacs, liver, and other organs) as well as swabs from egg nests and hatcheries.

2.3. Isolation and prevalence identification

The obtained samples were inoculated under aseptic conditions into buffer peptone water and incubated aerobically at 37°C for 24 hours. A loopful of the medium was streaked onto nutrient agar (Himedia, India) and blood agar. Suspected colonies were microscopically examined by Gram`s stained fixed films. Colonies that appeared as Gram positive cocci that arranged in grapes were suspected as Staphylococci. For identification of *S. aureus*, suspected colonies were further cultured on onto mannitol salt agar (Oxoid, UK) medium, and all plates were incubated aerobically at 37°C for 24 hours. *S. aureus* appears as yellow colonies and change the color of the media to pink.

2.4. Biochemical identification of *S. aureus*

2.4.1. Catalase test

Utilize a loop or sterile wooden stick to transfer a tiny quantity of colonial growth onto the surface of a clean, dry glass slide. Apply a drop of 3% H2O2 over the glass slide, then observe for the release of oxygen bubbles [11].

2.4.2. Coagulase Test

The coagulase test is performed to specifically identify *S. aureus,* which produces the enzyme coagulase that clots plasma. In a slide coagulase test, a loopful of the staphylococcal culture was emulsified in a drop of water on slide microscope and a loopful of rabbit plasma was added, then mixed well clumping within one or two minutes indicate positive reaction. In the tube coagulase test, bacterial suspension is mixed with rabbit plasma and incubated; the formation of a visible clot within 4–24 hours confirms a positive result, distinguishing *S. aureus* from coagulase-negative staphylococci [11].

2.5. Phenotypic characterization of biofilm formation:

2.5.1. Tube method

Tube method was done as described by Christensen et al., [8] for qualitative assessment of biofilm production. BHI broth with 2% sucrose (10 mL) in plastic tubes was inoculated with a loopful of microorganism from overnight culture plates. Tubes were incubated aerobically at 36°C ±1 for 24 hours. Tubes content was discarded, and tubes were washed once with 9 ml phosphate buffer saline pH 7.2 and then discarded. For biofilm fixation, 10 mL of freshly prepared sodium acetate (2%) was added to each tube for 10 minutes and then discarded. For biofilm staining, 10 mL crystal violet (0.1%) was then added to each tube, and tubes were left at room temperature for 30 minutes after which the stain was discarded. The washing step was repeated, and tubes were left to dry in an inverted position at room temperature. Biofilm formation was evaluated by the observation of visible film on the wall and bottom of the tube. In this test, biofilm formation was tested positive when a visible film was investigated along the inner wall and bottom of tube. Depending on this, isolates were scored as 0 for absence, + for weak, + + for moderate, and + + + for strong biofilm formation. The experiments were performed in triplicates and repeated three times.

2.6. Congo Red Agar method (CRA)

Qualitative method to detect biofilm production done by using a Congo red agar (CRA) medium [12]. CRA medium was prepared with brain heart infusion agar 37 g/L, sucrose 50g/L, and Congo red indicator 0.8 g/L. The first Congo red dye was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 min) separately from the other medium constituents. Later, it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. In this test, the Congo red dye was used as a pH indicator, showing black coloration at pH ranges between 3.0 and 5.2.

2.7. Antimicrobial susceptibility testing

The disc diffusion method was performed using 10 antimicrobial agents: Cefoxitin (CFX, 30 µg; MRSA indicator), levofloxacin (LEV, 5 µg), norfloxacin (Nor), chloramphenicol (C, 30 µg), rifampicin (RA, 5 µg), gentamycin (GM, 10 µg), and erythromycin (E, 15 µg), Ciprofloxacin(Cip,5 µg), Sulfonamides, Doxycycline (Do, 30 µg), (Oxoid, UK). The susceptibility testing and analysis of inhibition zones were performed in accordance with the Clinical Laboratory Standards Institute (CLSI) standards. Areference strain sourced from the Animal Health Research Institute in Dokki, Egypt, was utilized for quality control purposes. The categorization of isolates into multidrug-resistant (MDR) and extensively drug-resistant (XDR) classifications was performed based on the criteria set forth by Magiorakos et al., [13].

2.8. PCR detection of Virulence and resistance genes of *S. aureus*

PCR was performed to identify *S. aureus* by targeting the *16S*rRNA gene, and finding related virulence and antibiotic resistance genes. The primer sequences and PCR cycling conditions are specified in Supplementary (Table 1), with all primers obtained from Metabion (Germany). Genomic DNA was extracted utilising the QIAamp DNA Mini Kit (QIAGEN, Germany), and the purity and concentration of the DNA were evaluated with a NanoDrop ND-1000 spectrophotometer. Each PCR reaction was formulated to a final volume of 25 µL, comprising 12.5 µL of Emerald Amp GT PCR Master Mix (Takara, Code No. RR310A), 1 µL of each forward and reverse primer, 5 µL of DNA template, and 5.5 µL of PCR-grade water. Negative controls (lacking DNA) and positive controls (reference *S. aureus* strains from the Animal Health Research Institute, Egypt) were incorporated. Amplified products were analysed on a 1.5% agarose gel (w/v) using horizontal electrophoresis (AppliChem, Germany) and visualised with a gel documentation system (Alpha Innotech, Biometra).

Table 1 Oligonucleotide primers sequence of *S. aureus* used in cPCR

	Target gene	Sequence	Amplified product
Confirmatory gene	***16SrRNA***	AGTTTGATCCTGGCTCAG	228 bp
		GGTTACCTTGTTACGACTT	
Virulence genes	***clfB***	ACA TCA GTA ATA GTA GGG GGC AAC	205 bp
		TTC GCA CTG TTT GTG TTT GCA C	
	Eno	ACGTGCAGCAGCTGACT	302 bp
		CAACAGCATYCTTCAGTACCTTC	
	Coa	ATA GAG ATG CTG GTA CAG G	Four different types of bands may be detected 350 bp 430 bp 570 bp 630 bp
		GCT TCC GAT TGT TCG ATG C	
	Spa	TCA ACA AAG AAC AAC AAA ATG C	226 bp
		GCT TTC GGT GCT TGA GAT TC	
Resistance genes	***blaZ***	TACAACTGTAATATCGGAGGG	833 bp
		CATTACACTCTTGGCGGTTTC	
	mecA	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp
		CCA ATT CCA CAT TGT TTC GGT CTA A	
	tetK	GTAGCGACAATAGGTAATAGT	360 bp
		GTAGTGACAATAAACCTCCTA	
	vanA	CATGACGTATCGGTAAAATC	885 bp
		ACCGGGCAGRGTATTGAC	
	vanB	GTGACAAACCGGAGGCGAGGA	433 bp
		CCGCCATCCTCCTGCAAAAAA	

3. Results and Discussion

3.1. Prevalence of bacterial isolates from samples collected:

A total of 130 (49.8%) isolates were obtained from the 261 investigated samples, which were taken from the lungs, air sacs, yolk sacs, and other internal organs of deceased embryos, as well as swabs from egg nests and hatcheries. The samples were collected from different districts in Egypt (Table 2)

3.1.1. Identification of S.aureus isolated from dead in shell eggs:

A bout 130 isolates produces yellow colonies with yellow zones on mannitol salt agar medium. All suspected Staphylococcus species positive to catalase test. The coagulase test revealed 69% of isolates were positive to tube coagulase, while 57% of isolates were positive to plate coagulase.

Table 2 Distribution and frequency of suspected bacterial isolates in different regions under investigation.

Suspected bacterial isolates	Dakahlia I	Ismalia	Gharbia	Dakahlia II	Total isolates	%
S. aureus	55	40	15	20	130	49.8%

3.2. Phenotypic characterization of biofilm formation of *S. aureus*

The tube method was applied on a represented 50 isolates of *S. aureus*. The results revealed that 32 (64%) isolates where 12 isolates were strong, 8 moderate, 12 weak and 18 negative for biofilm formation.

3.3. Antibiogram profile of *S. aureus*

According to CSLI 2022 interpretive categories and zone diameter for S. aureus, it was detected that the examined S. aureus isolates were resistant to Erythromycin, Rifampim by percentage (100%), Chloramphenicol (88%), Sulfonamides (76%), Doxycyclines (62%), Cefoxitin (50%) and identified as MRSA, Norfloxacin (50%), Levofloxacin and Ciprofloxacin (24%) and Gentamycin (12%), table (2). Out of 50 tested isolates, 22 isolates were XDR, 28 isolates were MDR. The AMR index ranged between 0.9 and 0.2 as show in Table 3.

Table 3 Antimicrobial susceptibility of *S. aureus* across different antimicrobial classes.

Antimicrobial class	Anti-microbial target	Result			
		Resist		Sensitive	
		No	%	No	%
Macroloides	Erythromycin (E15)	50	100	-	-
Tetracycline	Doxycycline (Do30)	31	62	19	38
Fluroquinilones	Levofloxacin (Lev5)	12	24	38	76
	Ciprofloxacin (Cip5)	12	24	38	76
	Norfloxacin (Nor)	25	50	25	50
Cephalosporins	Cefoxitin	25	50	25	50
Sulfonamide	Sulfa+trimethoprim (sxt)	38	76	12	24
Amphenicols	Chloramphenicol (C30)	44	88	6	12
Ansamycin	Rifampim(RA5)	50	100	-	-
Aminoglycosides	Gentamycin(G10)	6	12	44	88

3.4. Molecular characterization of *S. aureus*

PCR results revealed that all tested isolates were successfully amplified the *16s*rRNA gene. In addition to the PCR analysis of *eno*, *spa*, *clf*B genes was performed for the virulence gene profiling. All isolates (100%) exhibited the *eno*

gene, the *spa* gene was confirmed in 71.4% of the isolates, while the *clf*B gene was found in 57% of the tested isolates. According to the phenotypic antimicrobial resistance profile, *bla*Z, *mec*A, *tet*K, *van*A, and *van*B genes were characterized molecularly. The *bla*Z gene for β-lactam resistance was successfully amplified in 85.7% of the respective isolates, All isolates (100%) carried the *mec*A gene, which indicates the methicillin resistance. Likewise, the *tet*K gene (confers resistance to tetracycline) was detected in 100% of isolates. In the other hand, the gene *van*A, which is associated to vancomycin resistance, was found in 42.8% of the tested isolates, while *van*B gene was not detected in the all examined isolates. This finding signifies dissimilar distribution of the resistance genes, all were positive in *mec*A and *tet*K but all negative in *van*B (Table 5).

S. aureus is a major pathogen causing great losses in poultry production. The presence of this bacterium in poultry environments, particularly in fertilized eggs, which lead to late embryonic death and loss huge numbers of newly hatched chicks. Beside to it is alarming from a public health perspective. In this study, the molecular and bacteriological characteristics of *S. aureus* strains from dead-in-shell eggs are examined in order to detect virulence and antibiotic resistance genes that may cause embryo dead as well as food contaminations. Characterization of the genetic profile of these isolates is essential to enhance biosecurity, establish antibiotic resistance monitoring programs and minimize the risk of transmission of virulent strains through the food chain **[5, 6]**.

Table 4 Antimicrobial resistance pattern of *S. aureus* isolates

No of tested isolates	No of Antimicrobial used	No of resistant isolates/ total antimicrobials	AMR index
50	10	*7 (9/10)*	(XDR) 0.9
		15 (8/10)	(XDR) 0.8
		2 (7/10)	(MDR) 0.7
		6 (6/10)	(MDR) 0.6
		6 (5/10)	(MDR) 0.5
		4 (4/10)	(MDR) 0.4
		8 (3/10)	(MDR)0.3
		2 (2/10)	0.2

This study identified *S. aureus* as the primary bacterial pathogen found in dead-in-shell eggs, accounting for 49.8% (130 out of 261) of the total bacterial isolates. Fujimotoet al., [14], identified bacterial pathogens from eggshells, egg yolks, feed, and air samples in chicken houses in 43% of tested samples. The findings of the present study align with those of Fujimotoet al., [14], which showed high incidence of *S. aureus* in 49.8% of tested samples) highlighting the pervasive occurrence of bacterial contamination in poultry-associated habitats. The significant prevalence of *S. aureus* in various studies and geographies indicates that continuous efforts are essential to enhance biosecurity, sanitation, and monitoring measures to mitigate the risk of bacterial contamination in poultry production and handling. Sharan et al., [10] indicated that 40% of *S. aureus* isolates from chicken eggs exhibited strong biofilm formation, 30% moderate, 20% weak, and 10% none. Their elevated proportion of biofilm producers may indicate variations in environmental circumstances or strain pathogenicity. Fujimotoet al., [14] investigated the biofilm-forming abilities of bacterial strains obtained from the egg surfaces of lake sturgeon, revealing that over 50% of the isolates exhibited high biofilm formation, 30% moderate, and 20% weak. Their study, which examined various species, noted that a substantial proportion of environmental bacterial isolates (50%) can develop biofilms. This finding corroborates the current study's conclusion that biofilm formation is a prevalent characteristic among bacterial isolates from eggs, albeit with differing specific percentages attributable to environmental influences and species variations.

The phenotypic antibiogram profiles of the isolates provide crucial insights into the antimicrobial resistance patterns of the bacteria. Of the S. aureus tested isolates, 52% were classified as MDR, demonstrating resistance to over three antimicrobials from various classes, whereas 44% were classified as XDR. A complete resistance of 100% was noted for erythromycin and rifampin, signifying a significant level of resistance to these frequently utilized antibiotics. Additionally, the research conducted by AbdelRahman et al., [15] demonstrated that all isolates (100%) of *S. aureus* exhibited resistance to penicillin G, ampicillin, and cefoxitin. In the present investigation, resistance to doxycycline was detected in 55% of the isolates, whereas, AbdelRahman et al., [15] reported a resistance rate of 85.7%. Approximately

50% of the *S. aureus* isolates exhibited resistance to cefoxitin and were classified as MRSA. The slight difference in percentage due to the difference in total numbers of tested isolates.

The amplification of the *16S* rRNA gene in *S. aureus* isolates was conducted to validate its presence. The *16S* rRNA gene is a highly conserved genetic marker commonly used for the identification of *S. aureus*. In this study, all *S. aureus* isolates successfully amplified the *16S* rRNA gene. This finding aligns with previous research, including Ariyanti et al. [16] who achieved 100% amplification in food-derived isolates, and Kim et al., [17] who reported a 95% success rate in meat and milk samples. Bhunia [18] also confirmed the gene's reliability for PCR-based detection of *S. aureus,* supporting its broad application in both clinical and food safety diagnostics. The PCR approach was employed to detect specific virulence genes in the verified bacterial isolates. Virulence genes encode proteins that are crucial for successfully establishing a bacterial infection in the host organism.

Table 5 Genotypic characterization of *S. aureus* isolated from dead in shell eggs:

	Target gene	**Total isolates**	**Positive**	**%**
Confirmatory gene	*16S*rRNA	20	20	100%
Virulence genes	*clf*B	7	4	57.1%
	Eno	7	7	100%
	Spa	7	5	71.4%
Resistance genes	*bla*Z	7	6	85.7%
	*mec*A	7	7	100%
	*tet*K	7	7	100%
	*van*A	7	3	42.8%
	*van*B	7	0	0%

The virulence genes of *S. aureus* significantly contribute to mortality in shell eggs. The *eno* gene, responsible for biofilm formation, was successfully amplified in 100% of the tested isolates. This result showed totally agreed with Wong et al., [19] which detected *eno* gene in all isolates. The identification of the *eno* gene in all isolates indicates an augmented ability for tissue invasion, as this gene encodes enolase, which is implicated in glycolysis and plasminogen binding. This discovery aligns with the research conducted by Wong et al., [19] which identified a significant prevalence of the *eno* gene in human-associated MRSA isolates.

The *spa* gene, which allows *S. aureus* to evade the host immune response, was amplified in 71.4% of the isolates. This finding is in agreement with AbdelRahman et al., [15] who reported the presence of the *spa* gene in *S. aureus* strains isolated from ducks, indicating its common occurrence in poultry-associated environments. The presence of the *spa* gene is significant as it contributes to immune evasion, allowing the pathogen to persist in host tissues and potentially cause systemic infections. Its detection in the majority of isolates highlights the pathogenic potential of the strains involved in embryonic mortality in shell eggs and emphasizes the need for targeted control strategies in hatchery settings. The identification of the *spa* gene verifies the existence of staphylococcal protein A, which plays a role in immune evasion. This discovery corresponds with the research conducted by Plano et al., [20] who similarly detected the *spa* gene in *S. aureus* isolates derived from animal-origin meals. The consistency of findings across research suggests that *spa* is a prevalent virulence factor in *S. aureus* strains, enhancing their capacity to circumvent the human immune response. Identification of the *clf*B gene in 57.1% of isolates, which is essential for the adhesion of *S. aureus* to fibrinogen. This discovery aligns with the research conducted by AbdelRahman et al., [15] who similarly detected the *clf*B gene in a substantial percentage of *S. aureus* isolates from ducks.

For resistance genes *mec*A gene was successfully identified in 100% of the tested *S. aureus* isolates, which are critical markers for MRSA (Methicillin-resistant *Staphylococcus aureus*). Which resist the majority of the antibiotics used to treat *S.aureus* and lead to great losses in poultry industry. The ability of MRSA to form biofilms, as shown in the study by [14, 21], enhances its survival on surfaces and equipment, contributing to its spread within poultry systems. The presence of MRSA in poultry not only affects animal health and productivity but also poses a potential risk to humans through direct contact or consumption, reinforcing the need for continuous monitoring, prudent antibiotic use, and stringent hygiene protocols throughout the poultry production chain. This discovery aligns with Fermin et al., [22]

results, which identified *mec*A in a substantial percentage of MRSA strains obtained from clinical and environmental specimens and AbdelRahman et al., [15] who characterized MRSA strains isolated from ducks, revealing the presence of multiple toxin and antibiotic resistance genes, underscoring the virulence and resilience of these strains in poultry environments.

The identification of the *bla*Z gene in 85.7% of examined isolates indicates resistance to beta-lactam antibiotics, such as penicillin. This corresponds with the results of Morshdy et al., [23] who documented a significant prevalence of the *bla*Z gene in *S. aureus* isolates from ready-to-eat food in Egypt. In their research, 73.3% of isolates possessed the *bla*Z gene, aligning with our detection rate and underscoring the apprehension of beta-lactam resistance in *S. aureus.* The detection of the *tet*K gene in all isolates signifies resistance to tetracycline drugs, corroborating findings by Kisa et al., [24] who reported a comparable incidence of *tet*K in *S. aureus* isolates from poultry flesh. This gene, which encodes an efflux pump mechanism, is extensively disseminated across *S. aureus* strains, hence complicating the treatment of infections caused by these resistant strains. In Turkey, Kisa et al., [24] identified *S. aureus* with resistance genes like *tet*K in poultry meat, while Fazelnejad et al., [25] and Momoh-Zekeri et al., [26] provided molecular evidence of antibiotic resistance and virulence factors in poultry-associated *S. aureus*, indicating that these bacteria can persist and evolve in food chains.

The identification of the *van*A gene in 42.8% of examined isolates is especially alarming, as it imparts resistance to vancomycin, a critical last-resort antibiotic. *S. aureus* strains which harbor *van*A gene and MRSA responsible for dead in shell embryos, omphalitis and mortality in newly hatched chicks. This outcome aligns with research conducted by Fazelnejad et al., [25] which identified the *van*A gene in *S. aureus* isolates from several sources, underscoring the significant concern of vancomycin resistance. *van*A gene was detected in 54.4% of isolates in the study of Shaimaa et al., [27]. Similarly, Hizlisoy et al., [21] and Morshdy et al., [23] found vancomycin resistance genes in foodborne *S. aureus*, highlighting the role of animal-derived foods in the dissemination of resistance determinants. Moreover, Momoh-Zekeri et al., [26] emphasized the zoonotic risk associated with resistance genes in *S. aureus* from non-clinical sources, including poultry. The detection of *van*A in poultry-associated isolates signifies an urgent need for strict antibiotic stewardship, routine molecular surveillance, and biosecurity interventions in poultry farms to prevent the spread of such highly resistant strains into the community and clinical settings.

No amplification of the *van*B gene was detected, indicating that the isolates lack this gene. This absence aligns with the observations of Momoh-Zekeri et al., [26] who reported that *van*B is less frequently detected in *S. aureus* isolates than *van*A. The absence of *van*B in our isolates suggests that vancomycin resistance in these bacteria is probably attributable to the *van*A gene, which functions via an alternative mechanism.

This study underscores the substantial bacterial contamination in poultry-related habitats, specifically emphasizing dead-in-shell eggs, hatcheries, and egg nests. Identified key pathogens include *S. aureus*, exhibiting alarming levels of drug resistance. The investigation indicated significant biofilm-forming capabilities among the isolates, dependent on the interaction between bacterial cells, substrates, and the surrounding media, enabling them to avoid the immune response and exhibit great resistance to traditional antimicrobials. The virulence genes played a crucial role in this study, facilitating the microorganism's adhesion to eggshells, subsequently penetrating the shells to reach the embryos and induce omphalitis and yolk sac infection in chicken embryos, thereby underscoring the challenges of persistence and resistance.

Management of the spread of antimicrobial resistant pathogenic bacteria, such as *S. aureus* in poultry production represents an important public health and economic issue. Infection of poultry with MDR strains not only effect on animal health and productivity, but also pose serious risk to human health via food chain, environmental pollution and occupational exposure. Appropriate control strategies are based on good biosecurity measures, active monitoring of resistant strains and antibiotics prescription based on antibiotic susceptibility testing. Furthermore, increased biosecurity practices at the hatcheries, farms and application of alternative interventions (probiotics, vaccine, bacteriophages) may reduce the burden of resistant bacteria. Education and training of farm staff, and veterinaries, are also essential for achieving a responsible use of antibiotics throughout the poultry sector. It is highly advisable to augment biosecurity protocols in chicken hatcheries and egg production facilities to reduce bacterial contamination and transmission. Consistent surveillance and judicious application of antibiotics must be implemented to reduce the emergence of antimicrobial resistance. Furthermore, extensive molecular surveillance is recommended to swiftly identify evolving resistance and pathogenicity patterns.

4. Conclusion

This study established that *S. aureus* is a frequent infection linked to dead-in-shell eggs, demonstrating considerable biofilm production ability and widespread antibiotic resistance, including MRSA. Molecular investigations indicated a

pervasive occurrence of essential virulence genes (*eno, spa, clfB*) and resistance genes (*bla*Z, *mec*A, *tet*K, *van*A), highlighting the poultry industry threat associated with contaminated fertilized eggs, hatcheries and poultry farms.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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