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College of Veterinary Medicine

Epidemiological study of *Staphylococcus aureus* and the related virulence genes from chicken farms

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Supervised by

Prof. Dr. Dhyaa Mohammad Taher Jwher

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Epidemiological study of *Staphylococcus aureus* and the related virulence genes from chicken farms

A Thesis Submitted

by

Ramadhan Fatah Miro Abdel Rahman

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Prof. Dr. Dhyaa Mohammad Taher Jwher

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

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God is the source of success Researcher

Ramadhan Fatah Miro

Abstract

Despite receiving great attention to the poultry industry in Iraq, it is still affected by various problems that have led to great economic losses. Including *Staphylococcus aureus* (*S. aureus*), which causes invasive diseases such as arthritis and septicemia, as well as food poisoning in humans. This study aimed to assess the levels of biosecurity measures in poultry farms by evaluating the prevalence of *S. aureus* contamination in broiler farms, determination of Methicillin-resistant *Staphylococcus aureus* (MRSA), provide epidemiological data regarding the overall occurrence of this microorganism in the chickens and its surrounding environment and detection of virulent gene of the isolates.

The study randomly targeted 26 broiler farms in the governorates of Duhok, Nineveh, and Erbil in northern Iraq. A questionnaire form was adopted to investigate the application of biosecurity procedures in poultry farms within the different study areas, for three main axes including: cleaning and disinfection, isolation, and monitoring, during the period from September 2024 to January 2025, It also collected (234), swabs and samples, including workers hand, chicken (skin), ventilator, feeder, water source, soil, bedding, grass and chicken feed, from each farm, and then transferred directly to the Scientific Research Laboratory at the College of Veterinary Medicine/University of Mosul for necessary bacteriological laboratory tests.

Traditional methods were used for isolation and identification of *S. aureus* including culture on differential media and biochemical tests. Chromogenic agar culture was used to diagnose MRSA, while molecular methods were used to detect the *nuc* gene to identify *S. aureus* and the *mec A* gene to identify MRSA. Pearson correlation test was used to find the relationship between the applied biosecurity measures and isolation rate.

The virulence factors *hlg, ebpS, fnbB* and *ica* genes were also investigated among MRSA isolates.

The isolation results of *S. aureus* showed that (101) samples out of (234) samples were positive, i.e. (43.16%) of the total samples included in the study (workers hand, chicken, ventilator, feeder and water, soil, bedding, grass and chicken feed). The highest isolation rate was from workers' hand, chicken at 53.85%, and the lowest isolation rate was from feeder and water at 34.61%, The highest prevalence rate of *S. aureus* was recorded among samples from different fields and from areas, from workers' hands and chickens at 13.86%, while the lowest prevalence rate was in the feeder and water samples at 8.91%.

The highest prevalence rates were recorded in Duhok Governorate, followed by Erbil and then Nineveh, at a rate of 50.50%, 37.62%, and 11.88%, respectively.

Regarding biosecurity measures, 19 categories were applied in No. 6 at the age of 7 days, representing 86.36% of the categories, and no isolation rate was recorded. In contrast, field No. 22, which is 40 days old, 5 categories were applied, representing 22.73%, and an isolation rate of 100% was recorded in the samples studied.

The results of MRSA isolation showed that 38 samples out of 101 samples of *S. aureus*, were positive, *i.e.* 37.62%

The highest prevalence rates of MRSA were recorded among the samples of different fields and from different areas, from the bedding and chicken at 23.68% and 18.42% respectively, while the lowest prevalence rates were in the Grass, Soil and Chicken feed samples at 2.63%, 5.26% and 5.26% respectively.

The study also revealed the presence of *fnbB, ica A, hlg,* and *ebp S* genes at rates of 63.2%, 57.9%, 60.5%, and 50%, respectively, of 38 MRSA isolates from different sources.

The high isolation rates of *S. aureus* and MRSA, along with their virulence genes, indicate their potentiality to cause infections that may be difficult to treat, and all components of the poultry environment under study are considered potential reservoirs.

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List of abbreviations

Abbreviation	Full Name
S. aureus	*Staphylococcus aureus*
SSSS	Staphylococcal Scalded-Skin-Syndrome
ClfA	Clumping factors A
ClfB	Clumping factors B
Bbp	Bone sialoprotein
AdsA	Adenosine synthase A
IsdABCH	Iron-regulated surface binding proteins
Can	Collagen adhesion
FnBPs	Fibronectin-binding proteins
eDNA	Extracellular Deoxyribonucleic acid release
icaADBC	Expression of intercellular adhesin operon
MRSA	Methicillin-resistant Staphylococcus aureus
kD	Kilodaltons
AA	Amino-Acid protein
PCR	Polymerase Chain Reaction
°C	Celsius temperature or centigrade
CLSI	Clinical and laboratory standards institute
et al.,	And others
FC	Fragment crystallizable region
IgG	Immunoglobulin G
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
TBE or Tris Borate EDTA	Tris base, boric acid and EDTA
EDTA	Ethylenediaminetetraacetic acid
TE Buffer	Tris-EDTA buffer
Nuc	Nuclease
mecA	Methicillin-resistance encoding gene
Hlg	Hemolysin gamma gene
ebpS	Elastin-binding protein S
fnbB	Fibronectin-binding proteins
ica A	Intercellular Adhesion gene A
MSA	Mannitol Salt Agar
MDR	Multi-drug resistance
HA-MRSA	health care-associated- Methicillin-resistant *Staphylococcus aure*us
SCCmec	Staphylococcal Cassette Chromosome mec

Chapter One
Introduction

1-1: Introduction:

Staphylococcus aureus (*S. aureus*) is one of the most important pathogens that threaten human life due to the high rates of infection and mortality it causes, despite its symbiotic presence with living organisms (Ramana *et al.*, 2009; Pollitt *et al.,* 2018). The bacterium is highly tolerant of physiochemical environmental conditions, as it can survive in the open air for several days to weeks, moving long distances, and is tolerant of drought (Carter and Cole, 1990; Kloos and Bannerman, 1995). It can also remain alive for several weeks in dry pus (Edmond *et al.*, 1996). It can withstand a temperature of (60) °C for (30) minutes, but it dies after (60) minutes at the same temperature (Koneman *et al.*, 1997a).

One of the most important sources of persistent infection with *S. aureus* is domesticated animals, which scientists have classified as reservoirs for the bacteria, in addition to being a potential source of infection for other animals and humans through its colonization and continuous presence on the mucous membranes of the nose, udder, teats and vagina, as noted by (Capurro *et al.,* 2010). The skin and tonsils of pigs, chickens, and turkeys often harbor bacteria and are potential sources of *Staphylococcus aureus* contamination (Pal *et al*., 2020).

Infection occurs in various types of birds, especially chickens, turkeys, ducks and geese, and occurs in the form of yolk-sac infection in embryos or chicks. It also takes the form of acute septicemia in adult chickens, which develops into chronic arthritis or air-sac infections.

Bumblefoot is a term used to describe local inflammation affecting the legs of chickens (Mohammadali Tabar *et al.*, 2024).

The most important sources of contamination in poultry farms with microbes are: workers, human waste, drinking water, feed, tools used in the field, rodents and hatcheries (Okonko *et al.*, 2010; Begum *et al.*, 2023). In addition to the environment in which poultry are raised, such as water, soil, bedding, feces, waste, sick and dead birds, eggs, and other poultry products (Hossain *et al.*, 2008; Eja *et al.*, 2012; Adeyanju and Ishola, 2014; Igbinosa, 2014; Khan *et al.*, 2014; Laban *et al.*, 2014).

Transmission occurs in several ways, including inhalation of air, consumption of contaminated water and food, direct contact through hands or contacts with secretions or contaminated materials and vectors (Cuny *et al.*, 2010; Ferreira *et al.*, 2011). Several studies conducted on poultry farms had revealed the presence of *S. aureus* in samples collected from humans, chickens, rodents, poultry litter, and soil surrounding the farm (Suleiman *et al*., 2013; Assafi *et al*., 2020; Sonola *et al*., 2023).

S. aureus has been associated with several conditions including dermatitis, omphalitis, femoral head necrosis, arthritis, tendinitis, and Bumblefoot (Suleiman *et al.*, 2013; Abd El-Tawab *et al.*, 2018; Cheung *et al.*, 2021), Many studies on poultry, including live chickens, sick chickens and dead chickens, had confirmed the presence of *S. aureus* in significant quantities (Sonola *et al.*, 2023).

Direct contact among poultry farm workers during field management operations is an important factor in the transmission of *S. aureus* from poultry to farm workers and vice versa (Assafi *et al.*, 2020). Thus, *S. aureus* isolated from poultry is considered a global risk indicator for humans living near poultry farms or who deal with them, whether in the field or through their production chains (Wertheim *et al.*, 2005). This is also due to the failure of cleaning and sterilization operations for the fields and their

components and their contamination with chicken remains, or through handling birds for therapeutic purposes. On the other hand, an epidemiological study revealed the presence of bacteria at a rate of 50% in air samples from inside poultry fields, which is a cause of water and food contamination for workers (Thompson *et al.*, 1980; Hussein *et al.*, 2015). Thus, the reverse transmission from workers can also cause contamination and transmission of *S. aureus* to birds and their environment if biosecurity procedures are not applied correctly (Hussein *et al.*, 2015).

Several studies conducted on poultry had previously documented different rates of *S. aureus* isolated from the respiratory tract, its secretions and eye secretions, some of which showed signs of conjunctivitis, facial edema, and respiratory infections (Abdellatif *et al.*, 2018). Additionally, *S. aureus* has been isolated from egg surfaces (Salihu *et al.*, 2015). Feed is often obtained from different sources and locations, so it remains a potential source for bacteria to enter poultry farms through the various processes that are carried out on it, starting from harvesting and ending with packaging and marketing (Okoli *et al.*, 2005; Chowdhuri *et al.*, 2011). Drinking water from various sources is contaminated with microbes as a result of bird behavior, feed, breeding system, feces, mismanagement and workers (Folorunso *et al.*, 2014; Oviasogie *et al.*, 2016) The number of microbes increases in poultry farm floors, including *Staphylococcus aureus*, during the progress of breeding periods (Folorunso *et al.*, 2013). Soil and its surroundings are contaminated by contact with human and chicken waste when dropped into the environment (Gómez *et al.*, 2014; Silva *et al.*, 2021).

The purposes of this study are:

- Isolate and characterize the *S. aureus* and MRSA in chicken and their environment.

- Study the relation between biosecurity measures and the level of Staphylococcus aureus contamination.
- Provide epidemiological data regarding the overall occurrence of this microorganism in the chicken production chain.
- Detection of virulent genes of the isolates.

Chapter Two

Review of literature

2-1: Staphylococcus:

The bacterium was discovered in 1871 by Von, "a German scientist", who described it as balls in patients with kidney diseases and called it Micrococci. Later, the scientist "Bloroth" classified it in (1874) based on its cellular arrangement as monococcus, diplococcus, streptococcus, and gliacoccus (Humphreys, 2002; Bhunia, 2008). In 1880, both Austin, a Scottish surgeon, and Pasteur, a French scientist, confirmed that these cocci had the ability to cause diseases. Later, Austin called them Staphylococci, a Greek word composed of two parts: staphyle, meaning a bunch of grapes, and kokkos, meaning berry (Humphreys, 2002; Ryan and Ray, 2004;).

One of the most important species belonging to the Staphylococcaceae family is *S. aureus*, which is a facultative anaerobic Gram-positive bacterium, with a diameter ranging between 0.5 and 1.5 micrometers, non-spore-forming and non-motile, forming groups similar to grape clusters, producing the catalase enzyme and not producing the oxidase enzyme (Asperger and Zangerl, 2011).

This bacterium colonizes body openings such as the nostrils, various mucous membranes, skin, and urinary and reproductive systems of many animals, birds, and humans, with the nostril being the most colonized site (Chen *et al.*, 2017), and has the ability to invade a variety of hosts tissues and survive and multiply within the host cells (Watkins and Unnikrishnan, 2020).

Furthermore, nasal colonization by *S. aureus* increases the risk factor for infection (Sakr *et al*., 2018), *S. aureus* is an opportunistic pathogen that can cause many different infectious diseases in animals and humans.

In recent years, interest in epidemiological investigations of these microorganisms has increased, due to the emergence and development of some resistant strains to a wide range of antibiotics as a result of their excessive consumption, which represents a threat to public health (Kral and Schwartzman, 1964; Lina *et al.*, 1999).

S. aureus is the main causative agent of many diseases, the most important of which are: mastitis in cattle, sheep, goats and horses, as well as dermatitis in sheep, goats and horses, purulent infections in cats and dogs, as well as lymphadenitis in sheep, and other diseases in birds (Foggie, 1947). It also causes a wide range of diseases in humans, including pneumonia, sepsis, infections of the skin, soft tissues, bones and joints, and endocarditis (Nandhini *et al.*, 2022).

2-2: Classification of Staphylococcus:

The genus Staphylococcus contains more than thirty-five species and twenty-one subspecies (Brooks *et al.*, 2016). It is naturally found on the skin and mucous membranes of humans and other living organisms (Breuer, 2001). It's present and commensal in the human nose and pharynx (Al-camo, 2001).

Staphylococci grow on many culture media, as they ferment many types of carbohydrates, and also secrete many pigments between white and dark yellow (Liu *et al.*, 2005). It is divided into two groups according to their production of coagulase enzymes (Woo *et al.*, 2001).

S. aureus is regarded as the most important type of staphylococci, which got its name from the pigmentation of its colonies during growth on culture media with golden yellow, due to carotenoid pigments. These colonies are round, smooth, elevated and shiny. *S. aureus* usually forms

colonies that are grey to dark golden yellow in colour. (Murray *et al.*, 2013).

On blood agar, *S. aureus* colonies are round, smooth and small, with 1 to 2 mm in diameter, often stained and surrounded by a zone of beta-hemolysis (Matthew, 2012). It gives positive results for free coagulase, phosphatase, DNase, gelatinase and negative results for oxidase (MacFaddin, 2000). According to the second edition of Bergey's manual of Systematic Bacteriology, Staphylococcus is classified within the family Staphylococcaceae (Garrity *et al.*, 2005) as follows:

Kingdom	Bacteria
Phylum	Bacillota
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	Staphylococcus
Species	*staphylococcus aureus*

2-3: Virulence factors of *S. aureus*:

All *S. aureus* isolates produce several virulence factors that contribute to their pathogenicity, many of which cause clinical diseases such as food poisoning. The severity of infection cannot be determined by a single virulence factor; rather it is the result of the cumulative effects of several factors (Kuipers *et al.*, 2016). These factors include:

2-3-1: First - Cell-related factors:

These factors include:

2-3-1-1: Adhesions

The pathogenic *S. aureus* has the ability to adhere to the external components present on the surfaces of host cells, which is an important factor responsible for its pathogenicity (Tung *et al.,* 2000).

This adhesion occurs through a group of molecules that can attract and adhere to the surfaces of the host cells. This is known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) (Patti *et al.*, 1994).

Each group of these molecules is specialized for the species and is divided into:

2-3-1-1-1: Fibrinogen-binding proteins MSCRAMM

This group of proteins plays an important role in injury in cases of wounds and arthritis (Palmqvist *et al.,* 2005).

2-3-1-1-2: Fibronectin-binding proteins MSCRAMM

This group is important in cases of infection with foreign bodies as well as in cases of infectious endocarditis and bone inflammation (Kuypers and Proctor, 1989; Johansson *et al.,* 2001).

2-3-1-1-3: Bone sialoprotein-binding proteins

This group of proteins is only present in bacteria that cause inflammation on the surface of bone cells and arthritis (Ryden *et al.,* 1989; Bremell *et al.*, 1991).

2-3-1-1-4: Collagen-binding protein

Collagen is one of the main components found in most tissues, so bacteria can adhere to collagen and is a major virulence factor for *S. aureus* (Patti *et al.*, 1994).

2-3-1-1-5: Elastin-binding protein

This group of proteins is considered an important factor because they are present in high proportions in the lungs, skin and blood vessels (Park *et al.,* 1996), so this protein has a major role in adhesion to these organs.

2-3-1-1-6: Broad-specificity-binding protein

These are proteins specialized for a specific part of the receptors found on the surfaces of host cells. This protein has the ability to facilitate the attachment of *S. aureus* to several cells present in the host, including the surfaces of bone cells, collagen, fibronectin, fibrinogen and vitronectin (McGavin *et al.,* 1993).

2-3-1-2: Protein A:

These proteins are found on the surface of *S. aureus*, giving them the ability to bind to the Fragment crystallizable (FC) crystallized part of the immunoglobulin G (IgG), and thus provide an immune response to the host against the bacteria (Johansson *et al.*, 2001).

On the other hand, these proteins "A" work defensively, as they have the ability to prevent the host's immune system from phagocytizing bacteria, by binding to immunoglobulin A (IgA), and the process of continued bacterial reproduction works to exhaust the IgA for its work, then the bacteria begin to increase the attack and reproduction on the rest of the cells and infection occurs (Washington *et al.*, 2006).

2-3-1-3: Capsular polysaccharides:

The polysaccharides of the bacterial capsule are considered important virulence factors for causing infection in the host, and more than 90% of *S. aureus* strains possess this factor (Thakker *et al.,* 1998K; ampen *et al.*, 2005).

2-3-1-4: Peptidoglycan and lipoteichoic acid:

It is one of the large peptidoglycan molecules that contain long sugar chains responsible for the protective and shape-maintaining properties of bacterial cell walls, they are components of the cell wall of Gram-positive bacteria (McGavin *et al.,* 1993; Romaniuk and Cegelski, 2018;).

2-3-1-5: Surface components involved in biofilm formation:

The major surface proteins are clumping factors A and B (*ClfA* and *ClfB*), bone sialoprotein (*Bbp*), adenosine synthase A (*AdsA*), iron-regulated surface binding proteins (*IsdABCH*), collagen adhesion (*Cna*), and fibronectin-binding proteins (*FnBP*s), which enable *S. aureus* to produce biofilms by several strategies, including expression of several surface proteins including MSCRAMMs, extracellular DNA (eDNA) release, and expression of intercellular adhesin (*PIA*) by the icaADBC operon. (Romaniuk and Cegelski, 2018; Wu *et al.,* 2024).

2-3-2: Second - Extracellular protein toxins

They are toxins secreted outside the bacterial cell and include:

2-3-2-1: Exfoliative toxins:

Exfoliative toxins cause Staphylococcal Scalded-Skin-Syndrome (SSSS) as they cause inflammation of the inner dermis of the skin, leading to the appearance of granular ulcers on the surface of the skin (Yamasaki *et al.,* 2005).

2-3-2-2: Hemolysins toxins:

Most strains of *S. aureus* produce hemolysins toxins which include: alpha, beta, gamma and delta, which differ according to the number of amino acids (Freer and Birkbeck, 1982; Nasaj *et al.,* 2020), the most important one is an alpha toxin, which can lyse red blood cells, and causes

necrosis of skin cells and a toxic effect on the surrounding nerves (Dinges *et al*., 2000).

As for beta toxins, they have an important property in diagnosing the bacteria, as they give a complete lytic range on blood agar, which indicates a positive diagnosis (Koneman *et al.,* 1997b).

2-3-2-3: Leucotoxins:

Leucotoxins are toxins that destroy the cell membrane, and they are on two types of unrelated proteins S and F, *i.e*. "Slow and Fast-acting" (Prevost *et al.,* 1995). They work together and synergistically to destroy the cell wall. These toxins are called Synergohymenotropic (Supersac *et al.,* 1993). Among its types is Panton-Valentine leukocidin, which also consists of two factors that cause a lytic reaction of macrophages and multinucleated cells in animals and humans (Cribier *et al.,* 1992). Thus, it facilitates the occurrence of multiple diseases, including dermatitis and necrotizing hemorrhagic pneumonia in immunodeficiency patients (Gillet *et al*., 2002).

Methicillin-resistant Staphylococcus Methicillin-resistant *S. aureus* (MRSA) is responsible for the production of the toxin Panton-Valentine leukocidin, so screening for this toxin is tantamount to detecting these strains (Vandenesch *et al.,* 2003).

2-3-2-4: Heat-resistant antigenic toxins Pyrogenic toxin superantigens:

This group includes:

2-3-2-4-1: Enterotoxins:

Enterotoxins are food poisoning proteins and are characterized by being soluble in water, with a molecular weight ranging between (26-35) Kd. They are polypeptide chains that contain large amounts of enzymes

associated with peptide molecules, including: tyrosine, glutamic acid, aspartic acid and lysine, (Bronner *et al.*, 2004). They are heat and ambient conditions resistant, can withstand boiling for 30 minutes, and these toxins maintains their toxic composition under 121°C for 28 minutes (Stewart, 2017).

Enterotoxins are divided based on their molecular weight and differences in the immune response to them based on serology into several types, which are (*A, B, C1, C2, C3, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, U* and *V*) (Wright *et al.*, 2000; Akineden *et al*., 2008).

Symptoms of food poisoning by *S. aureus* appear within 24-48 hours' maximum, and this is attributed to the resistance of intestinal toxins to digestive enzymes present in the stomach and the protein-degrading enzymes such as papaya, renin, trypsin, chymotrypsin, and pepsin (Bergdoll, 1983).

Jablonski and Bohach (2001) indicated that 50-70% of *S. aureus* strains can produce intestinal toxins in food contaminated with the bacteria at a temperature of (15-18) °C for a maximum period of 3 hours, where an amount of the toxin is produced that can cause food poisoning. It has been found that 100-200 nanograms of the toxin can cause vomiting in humans.

2-3-2-4-2: Toxic shock syndrome toxin type 1:

Toxic shock syndrome toxin type 1 is a pathogenic toxin that is produced by *Staphylococcus aureus*. It is characterized by its rapid onset and high temperature, with severe ulcers on the surface of the skin. The incubation period of the disease lasts from one to two weeks, and the disease is rarely fatal (Brooks *et al.*, 2007).

2-3-3: Third - Exoenzymes

These enzymes are secreted externally and include:

2-3-3-1: Coagulase enzyme:

This enzyme is the key to the initial diagnosis of staphylococcal bacteria. It is an external protein that can bind to prothrombin in the host's blood and clot the plasma, then form a complex called staphylo-thrombin. It works as a protease enzyme and converts fibrinogen into fibrin.

This enzyme can protect bacteria from the host's immune defenses, the most important of which is phagocytosis, causing a clot around the bacteria inside the host's body (McAdow *et al.*, 2012; Tam and Torres, 2019).

2-3-3-2: Hyaluronidase enzyme:

This enzyme facilitates the entry and rapid spread of bacteria by breaking down hyaluronic acid in the soft tissues of the host (Makris *et al.*, 2004; Ibberson *et al.*, 2016).

2-3-3-3: Protease enzyme:

Researchers believe that it has several functions, including its participation in the destruction of protein tissues and facilitating the entry of *S. aureus* and causing disease, in addition to its ability to weaken the host's defenses and protect *S. aureus* from antibiotics by disabling the active peptide substance that prevents the destruction of bacteria (Tam and Torres, 2019; Alonazi, 2024).

2-3-3-4: Lipase enzyme:

Lipase enzyme plays an important role in breaking down host enzymes to facilitate the entry and reproduction of bacteria by disrupting the phagocytosis process and its effect on the granular cells of white blood cells by changing the components of their cell wall, to limit their defensive function, in addition to affecting fatty acids and analyzing the host's fat cells. It is believed that its work is similar to the work of the protease enzyme (Tam and Torres, 2019; Sargison *et al.*, 2020).

2-3-3-5: Penicillinase:

Until 1941, more than 90% of staphylococcal isolates were sensitive to penicillin, the same year the antibiotic was first used clinically. However, staphylococci rapidly developed resistance to penicillin, primarily due to the ability of organisms to produce penicillinase (beta-lactamase), an enzyme that cleaves the beta-lactam ring from the penicillin molecule and, through its presence on transmissible plasmids, is passed on to subsequent bacterial generations (Murray *et al.*, 2003).

2-3-3-6: Staphylokinase enzyme:

It is a 136 AA bacterial protein that is expressed by some lysogenic strains of *S. aureus*. This enzyme is secreted by some strains of Staphylococcus and helps them to spread rapidly, penetrate host tissues and multiply inside the host body by affecting the action of both α-defensins and plasminogen, thus suppressing the host's immunity as they act as a bactericidal antibiotic and are produced by a type of white blood cells, which is neutrophils, thus making it easier for the bacteria to cause infection (Bokarewa *et al.*, 2006; Vakili *et al*., 2018).

2-4: Methicillin-resistant *S. aureus* (MRSA):

Methicillin-resistant *S.aureus* (MRSA) was found in 1970 in the milk of Belgian cows with mastitis (Devriese and Homes,1975). However, the MRSA status of dairy workers had not been investigated, and MRSA had since been reported in several species, including humans, sheep, cattle, Buffalo and birds (Qudduomi *et al.*, 2006; Tareen and Zahra, 2023; Rahma and Jwher, 2024). Historically, MRSA infections in field animals had involved strains that resemble strains isolated from human hospitals (Rich *et al.*, 2005), and the assumption was that the direction of spread was from humans to animals. However, this situation has rapidly changed, with MRSA strains thought to have evolved in animals and subsequently

colonized and infected humans (Van Belkum *et al.,* 2009; Taha *et al*., 2024).

MRSA infections in animals are most commonly skin and soft tissue infections (especially post-operative). Over one year in the UK, 1.5% (95 from 6519) of clinical specimens from infected animals showed MRSA, this group included 69 dogs, 24 cats, one horse, and one rabbit (Rich and Roberts, 2004).

MRSA also caused mastitis in dairy herds, and occasionally purulent dermatitis in milkers (Grinberg *et al.,* 2004). Cows with mastitis were most likely to harbour MRSA, which had been attributed to horizontal transmission of MRSA via the wet hands of carriers or infected milkmen, as well as to topical antibiotic selection for treatment of mastitis cases (Macário Ferro Cavalcanti, 2019).

2-5: Epidemiology of *S. aureus*:

S. aureus is one of the most important causes of diseases that threaten human life due to the high rates of infection and death it causes, despite its presence in a symbiotic manner with living organisms (Ramana *et al.,* 2009; Lakhundi, and Zhang, 2018; Pollitt *et al.,* 2018; Bagheri *et al.,* 2019).

It is found in the surrounding environment such as water, soil and air. It has been isolated from foods of animal origin, and is a major cause of many cases of food poisoning around the world (Friese *et al.,* 2013; Wang *et al*., 2018).

The bacterium is highly tolerant of physiochemical environmental conditions, as it can survive in the open air for a long period (days to weeks), is mobile over long distances, and is tolerant of drought (Carter and Cole, 1990; Kloos and Bannerman, 1995). It also has the ability to remain alive for several weeks in dry pus (Edmond *et al.,* 1996). It can

withstand a temperature of (60) °C for (30) minutes, but it dies after (60) minutes at the same temperature (Koneman *et al.*, 1997a).

Many domesticated field animals are considered reservoirs and sources of continuous infection for *S. aureus* due to their presence and continuous colonization of the mucous membranes of the muzzle, vagina, udder and teats. They have been classified by scientists as reservoirs for the bacterium, in addition to being a source of infection for other animals and humans (Capurro *et al.,* 2010; Tareen and Zahra, 2023; Rahma and Jwher, 2024).

Raw milk contamination and *S. aureus* infections of animals on dairy farms occur due to contamination of the surrounding environment from animals suffering from subclinical infection, as well as milk equipment and workers (Stewart, 2017).

The spread of infection to other uncontaminated places occurs through the transfer of infected animals. Contaminated cheese can also serve as a reservoir for *S. aureus* and spread to the surrounding environment (Taha *et al*., 2024). The skin and tonsils of pigs, chickens and turkeys often harbor bacteria that are often potential sources of *S. aureus* contamination (Pal *et al.,* 2020).

2-6: Staphylococcosis in chickens:

Staphylococcosis infection occurs in different types of birds, especially chickens, turkeys, ducks and geese, and occurs in the form of yolk-sac infection in embryos or chicks. It also takes the form of acute septicaemia in adult chickens and develops into chronic arthritis or air-sac infections. Bumblefoot is usually given to the local inflammation that affects the legs of chickens (Casey *et al.,* 2007; Olayinka *et al.,* 2010; Mohammadali *et al.,* 2024).

Arthritis can be defined as an inflammation of the serous membrane of the joints (synovitis) mainly and usually accompanies (Marcon *et al.*, 2019), this condition in the joint tendons and their accompanying sheaths. This condition usually occurs intermittently in broiler flocks. The bacteria may infect other parts of the bird's body such as the skin, yolk sac, heart, vertebrae and eyelid (Chaudhary *et al.*, 2018; Andreasen, 2020).

This infection causes granulomas in the liver and lung (Daum *et al.*, 1990; Mohammadali *et al.*, 2024), and also causes septicemia in laying birds, causing sudden death. This infection is mainly prevalent in hot regions, and its symptoms are similar to fowl cholera (Chahota *et al.*, 2001; Sato and El-Gazzar, 2020).

It was noted that the most common joints affected by *Staphylococcus aureus* were the hock joint, the joint between the shin bone and the foot, in addition to the small joints between the toes (interphalangeal joint), which leads to lameness and inability to move as a result of severe inflammation of the joint, and developed into prolonged lying down and inability to reach water and feed sources, which led to death as a result of weakness and severe emaciation (Karwowska *et al.*, 1998; Knowles *et al.*, 2008; Kierończyk *et al.*, 2017).

In addition to the injury to the thoracic and lumbar vertebrae, which led to indirect pressure on the spinal cord, it was noted that there were no apparent symptoms on the joint, especially in the early stages of the injury or in cases of mild injury, and upon examination, they found that there was opacity and an increase in synovial fluid (Kierończyk *et al.*, 2017; Mohammadali *et al.*, 2024).

In severe cases, inflammation and painful ulcers were observed when pressed, and there was a cheesy substance inside them in chronic cases (Koplak and Mehler, 2000). The inflammation might extend to the back of the leg (flexor tendons of the fingers) as well as the tendons above the

Achilles joint (extensor tendons of the fingers). In addition, it had been shown that the tendon sheaths and the surrounding tissues appeared swollen and congested in cases of real injuries (Ibrahim *et al.*, 2000).

2-7: *Staphylococcus aureus* contamination of chickens, eggs and the rearing environment in poultry farms:

The most important sources of contamination in poultry farms with microbes were field workers, human waste, drinking water, feed, tools used in the field, rodents and hatcheries (Okonko *et al.*, 2010; Begum *et al.*, 2023), In addition to the environment in which poultry are raised, such as water, soil and bedding (Eja *et al.*, 2012; Igbinosa, 2014), feces and waste (Khan *et al.*, 2014), sick and dead birds (Hossain *et al.*, 2008), eggs and other poultry products (Adeyanju *et al.*, 2014; Laban *et al.*, 2014) *Staphylococcus aureus* in food is attributed to its ability to be transmitted through the food chain via human activities, *S. aureus* can adhere to processing equipment, tools, and environmental surfaces (Song *et al.*, 2024), figure (2-1).

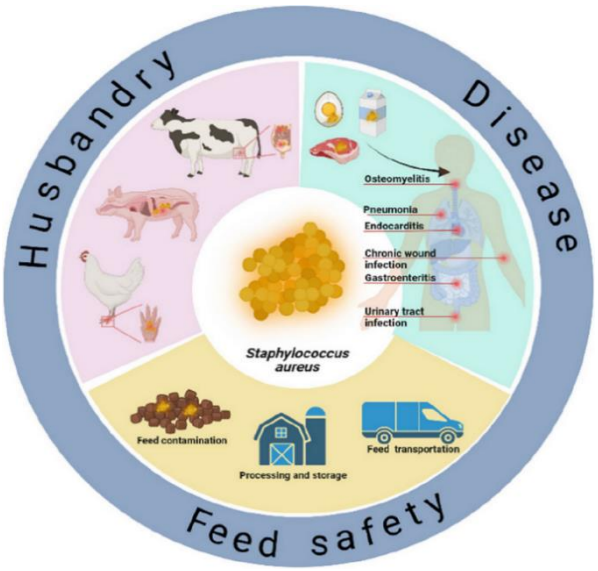


Figure (2-1): Transmission and risk factors of *S. aureus* in poultry and animal husbandry (Song *et al*., 2024).

S. aureus was transmitted by multiple routes, including inhalation of air, ingestion of contaminated food and water, direct contact with hands, or contact with secretions or contaminated materials and vectors (Cuny *et al.*, 2010; Begum *et al.*, 2023).

Different modes of transmission had been described, including direct contact, through hands, contact with secretions or contact with inanimate objects (infectious agents), ingestion of contaminated food and water, aerosols, and via vectors (Ferreira *et al.*, 2011; Adeyanju *et al*., 2014).

Several studies conducted on poultry farms had revealed the presence of *Staphylococcus aureus* in samples collected from humans, chickens, rodents, poultry litter, and soil surrounding the farm, Figure (2-2) (Adayel, 2005; Suleiman *et al.*, 2013; Assafi *et al.*, 2020; Sonola *et al.*, 2021).

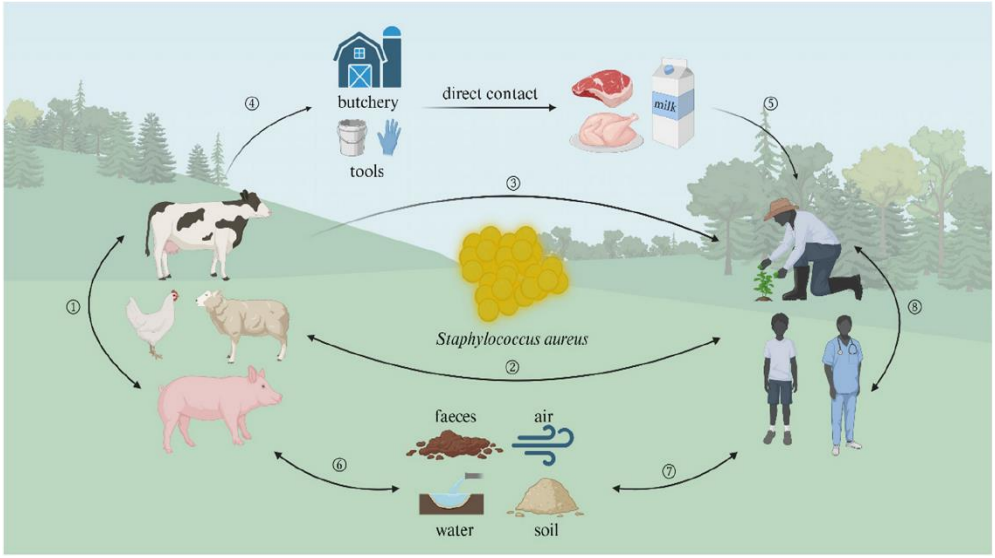


Figure (2-2): The transmission routes of *S. aureus* (Song *et al*., 2024).

The bacteria had been associated with several conditions including dermatitis, omphalitis, femoral head necrosis, arthritis, tendinitis, and

Bumblefoot (Suleiman *et al.*, 2013; Abd El-Tawab *et al.*, 2018; Cheung *et al.*, 2021),

In a study conducted in Italy on poultry fields and flocks over a period of one year, it was proven that *S. aureus* and methicillin-resistant *S. aureus* occurred at a rate of 23.8% (Pesavento *et al.*, 2007).

Many studies conducted on poultry, including live, sick and dead chickens, had confirmed the presence of *S. aureus* in large quantities (Bakheet *et al.*, 2014; Sonola *et al.*, 2023).

2-7-1: The role of workers in the occurrence of *S. aureus* contamination in poultry farms:

Many species of *S. aureus* coexist in humans and animals alike on the skin and mucosal surfaces such as the upper respiratory tract, gastrointestinal tract, and genitourinary tract (Todar, 2008; Weese, 2010).

The direct contact by poultry farm workers during field management operations was an important factor in the transmission of *S. aureus* and MRSA from broilers to farm workers and vice versa (Assafi *et al.*, 2020). Infection had been documented among broiler chickens and poultry farm workers, with 20% and 60% carriers and infected, respectively (Latour *et al.*, 2019). Thus, *S. aureus* isolated from poultry was considered a global risk indicator for humans living near poultry farms or who deal with them, whether in the field or through their production chains (Wertheim *et al.*, 2005). According to some studies, the percentage of isolation of the microbe from workers varied between poultry farms in different regions (CLSI, 2015; Conlon *et al.*, 2019).

It was closely linked to the high density of workers in the fields as well as the high density of birds within the field (Krupa *et al.*, 2014). This was also due to the failure of cleaning and sterilization operations for the fields and their components and their contamination with chicken remains, or

through handling birds for therapeutic purposes. On the other hand, an epidemiological study revealed the presence of *S. aureus* bacteria at a rate of 50% in air samples from inside poultry fields, which was a cause of water and food contamination for workers (Thompson *et al.,* 1980; Hussein *et al.,* 2015).

Thus, reverse transmission from workers could also cause contamination and transmission of *S. aureus* to birds and their environment if biosecurity procedures were not applied correctly (Hussein *et al.,* 2015).

2-7-2: *S. aureus* contamination of chickens:

Many studies conducted on poultry previously documented different rates of *S. aureus* isolated from the trachea, sinuses, eye and nasal secretions of poultry, some of which showed signs of conjunctivitis, facial edema and respiratory infections, with isolation rates ranging between 7.5% and 41.4% (Abdellatif *et al.,* 2018).

In addition, other studies indicated the isolation rates of *S. aureus* from the internal organs of poultry, especially from young ages, where it was isolated from the intestines, liver, heart, joints, and from the non-absorbed yolk sac, and it caused symptoms including bronchitis, rhinitis, and congestion of the chest and thigh muscles (Omar *et al.,* 2019).

Another study conducted in Duhok Governorate in northern Iraq on broiler chickens revealed an 84.8% bacterial isolation rate (Assafi *et al.,* 2020), another study conducted on laying hens in Nineveh Governorate recorded a bacterial isolation rate ranging from 62.5% to 79.16% (Shareef *et al.,* 2009). Previous studies indicated that *S. aureus* isolated from laying hens was more common than from broiler chickens (Pyzik and Marek, 2012).

2-7-3: Egg contamination with *S. aureus*:

The surfaces of chicken egg shells harbor many microorganisms that cause health problems for consumers, including *Escherichia coli*, *Bacillus, Salmonella, Listeria, and Streptococcus. S. aureus* constitutes a large and essential part of the microorganisms that had been isolated and studied from the surface of eggs (Mahdavi *et al.,* 2012; Salihu *et al.,* 2015).

Many scientists had pointed out the mechanism of shell contamination when eggs come into contact with contaminated surfaces at the beginning of egg laying (Smith *et al.,* 2000).

Several studies have been conducted to determine the prevalence of *S. aureus* on the surface of chicken eggshells. These studies reported contamination rates on eggshell surfaces were of egg contents contamination rates of 15.6%, 14.5%, 21.3%, 20.45%,19.33% and 10.16%, in Poland, Thailand, Egypt, Pakistan, Bangladesh and Burkina Faso, respectively (Pyzik *et al.,* 2014; Chaemsanit *et al.,* 2015; Eid *et al.,* 2015; Parveen *et al.,* 2017; Pondit *et al.,* 2018; Sawadogo *et al.,* 2023). Studies suggested that bacteria could penetrate the eggshell and contaminate its contents (Bahrouz and Al-Jaff, 2005).

2-7-4: Presence of *S. aureus* in feed:

Feed is a good nutrient for poultry, as it contains different types of proteins, both plant and animal, grains, in addition to amino acids, minerals, salts, and some food additives. It is mostly obtained from different sources and locations, so it remains a potential source for pathogens to enter poultry farms through the various processes that are carried out on it, starting from harvesting and ending with packaging and marketing (Okoli *et al.,* 2005; Chowdhuri *et al.,* 2011).

In a study conducted by researchers (Danbappa *et al.,* 2018; Faparusi, 2019) to determine the microbial load of types of contaminants associated with poultry feed in its manufacturing sites, where the presence of *S. aureus* was recorded in all tested feeds.

The relative humidity and temperature in improper storage and processing conditions and the failure to apply standards starting from the harvest and storage stage to the processing, packaging, marketing and healthy transportation stages were environmental factors that facilitated the growth and reproduction of living organisms in feed (Danbappa *et al.*, 2018).

Other studies had shown a high level of *Staphylococcus aureus* in feed that was contaminated with chicken droppings in the field (Thompson *et al.,* 1980; Sonola *et al*., 2021).

2-7-5: Presence of *S. aureus* in water:

Drinking water is one of the most important basic nutritional elements for poultry, as the quality of drinking water in poultry farms, regardless of its sources, whether from pipe water (drainage) or well water, is exposed to several risks due to its contamination with microbes as a result of bird behavior, feed, breeding system, feces, mismanagement and workers (Folorunso *et al.*, 2014; Oviasogie *et al.*, 2016).

As a result, the process of preparing high-quality water is essential for poultry farming, as large numbers of birds share the water source. Any impact on it might negatively affect the health and productivity of the birds, as it contributes to the transmission of many forms of parasitic, viral and bacterial infections (Zaman *et al.,* 2012).

The study conducted by researchers (Chat *et al*., 2019; Gyang *et al.*, 2019) revealed that water, soil, and bird remains were the most important sources of microbial contamination in poultry farms, which were the cause of food poisoning for consumers of meat contaminated with *S. aureus* during its transmission, marketing process and contamination of carcass tools (Omoya *et al.*, 2016).

The studies conducted by researchers (Maharjan *et al.*, 2017; Mantzios *et al*., 2023) revealed the presence of *S. aureus* bacteria in poultry drinking water at different rates.

The high temperatures and low water flow intensity in drinking water lines and systems had negatively affected the quality of water by creating suitable conditions for the sedimentation and accumulation of organic materials, solids and minerals, which facilitated and enhanced the growth of bacteria and the formation of biofilms, allowing them to remain in drinking water for a long time (figure 2-3) (Maharjan *et al.*, 2016; Maes *et al*., 2019).

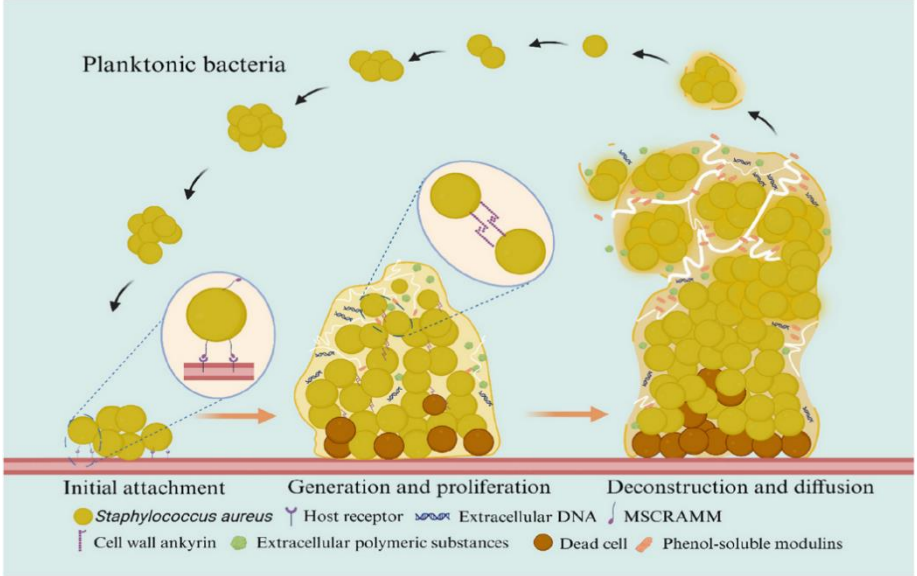


Figure (2-3): Biofilm formation stages for *S. aureus* (Song *et al.*, 2024).

2-7-6: Presence of *Staphylococcus aureus* in soil and litter:

The study conducted by researchers Folorunso *et al.* (2013) proved that the number of microbes in poultry farm floors, including *S. aureus*, increased during the progress of the rearing periods (Folorunso *et al.*, 2013; Sarkingobir *et al.*, 2020; Emmanuel-Akerele *et al.*, 2021).

The researchers (Sawadogo *et al.*, 2023) indicated a prevalence rate of *S. aureus*, which reached 51.78% in poultry litter samples. In another study conducted in Tanzania to estimate the prevalence of *S. aureus* in the north in the soil surrounding poultry housing, researchers (Sonola *et al.*, 2021) reported a rate of 24.5%, all of which were of the type resistant to antibiotics, as the soil served as a reservoir for them.

The contamination of soil and its surroundings increased based on contact with human and chicken waste when it was dropped into the environment (Gómez *et al.*, 2014; Silva *et al.*, 2021). Other studies had shown a high percentage of *S. aureus* in chicken waste inside the field (Thompson *et al.*, 1980; Sonola *et al.*, 2021).

2-7-7: Presence of Staphylococcus in air vents:

Air vents in intensive rearing conditions contain many airborne particles, including a mixture of biological materials from bacteria, toxins, gases, and organic volatile and adsorbed compounds, which are known as bio-aerosols (Hartung *et al.*, 2012; Ahmed *et al.*, 2013),

Its percentage increases with the presence of some contributing factors such as the accumulation of droppings on the litter, its dryness, and the speed of air currents inside the barn. Organic impurities also increase during the distribution of feed and in the moulting stage of birds in particular, which increases the chance of their deposition on airborne ducts (Wang *et al.*, 2010; Lima *et al.*, 2011).

The ventilation equipment in modern poultry and livestock farms had accelerated the exchange of air between the rearing barns and the external environment. A large amount of microorganisms, including *S. aureus*, were released with the exhaust air into the environment surrounding the poultry farms, which increased the contamination rates (Davies *et al.*, 1994; Duan *et al.*, 2008).

Some studies had indicated a direct relationship between the isolation rate of *S. aureus* from air vents and the level of contamination inside poultry farms (Duan *et al.*, 2009; Zhong *et al.*, 2009; Liu *et al.*, 2012).

2-8: Biosecurity in poultry farms

Biosecurity in poultry farms involved a set of fundamental practices and strategies. Those measures prevented the entry and transmission of pathogens in the farms, thus minimizing the negative impact they could have on poultry production (Abdelal *et al.*, 2016).

Biosecurity was an integral part of successful livestock and poultry production systems and refers to the measures or procedures followed to control and prevent the introduction and spread of pathogenic biological agents (Viruses, bacteria, fungi, parasites, etc.) into flocks. The failure of which led to the occurrence of diseases, reduced production and the destruction of flocks (Sharma, 2010; Assefa, 2022).

The components of biosecurity measures included sanitation, traffic control, isolation, periodic monitoring of flocks and vaccination (Assefa, 2022).

Chapter Three

Materials and Methods

3-1: Materials:

3-1-1: Laboratory devices:

Table (3-1): Laboratory devices used in the study.

No.	Device	Company	Origin
1	Microscope	Olympus CX21	Japan
2	Refrigerator	Beko	Turkey
3	Sensitive electric balance	Want	China
4	Water bath	Memmert	Germany
5	Incubator	Memmert	Germany
6	Autoclave	Hirayama	Japan
7	Hot plate stirrer	Worner lab	China
8	Microwave oven	LG	Korea
9	Eppendorf microcentrifuge	Wisd	Germany
10	Gel electrophoreses	Biorad	USA
11	Gel documentation system	Biorad	USA
12	Thermocycler	Biorad	USA
13	Distillation device	H2O lab	China
14	Vortex shaker	Thermo	USA
15	Centrifuge	Worner lab	China
16	UV Transillumination	Biometra	Germany

3-1-2: Laboratory equipments:

Table (3-2): Laboratory equipments used in the study.

No.	Equipment	Company	Origin
1	Plastic containers (50 ml)	Citrotest	China
2	Sterile plastic bags	Citrotest	China
3	Sterile cotton swabs	Citrotest	China
4	Graduated glass cylinders	Citrotest	China
5	Conical flasks	Citrotest	China
6	Test tubes	Citrotest	China
7	Disposable syringe	Shengguang	China
8	Micropipette	Dragon	China
9	PCR Tube 0.2 ml	Citrotest	China

10	Sensitivity disk test	Bioanalyse	China
11	Filter paper	Citrotest	China
12	Microscope slides	Citroglass	China
13	Bacteriological loop	Himedia	Indian

3-1-3: Cultural media:

Table (3-3): Culture media used in the study.

No.	Types	Company	Origin
1	Mannitol Salt Agar	Scharlau	Spain
2	Brain –Heart Infusion Broth	Himedia	Indian
3	Peptone water medium	Micro X press	Spain
4	Blood agar Base	Himedia	Indian
5	Agarose	Promega	USA
6	Agar agar	Himedia	Indian
7	MRSA Chromogen agar	Himedia	Indian

3-1-4: Chemicals and Reagents:

Table (3-4): Chemicals and Reagents used in the study.

No.	Types	Company	Origin
1	Hydrogen peroxide	Scharlau	Spain
2	Oxidase reagent	BDH	England
3	Gram stain	Scientific Atom	UK
4	Ethanol (70%)	Chem-lab	Belgium
5	Tris borate EDTA	Pomega	USA
6	Methyl-Red	BDH	England
7	Tetramethyl-phenylene Diamine Dihydrochloride	Scharlau	Spain
8	Master-mix	Promega	USA
9	Elution solution	Geneaid	Taiwan
10	Tris-Boric acid EDTA (TBE)	Geneaid	Taiwan
11	Red Safe dye	BioBasic	Canada
12	Ladder	Promega	USA

3-1-5: Special solutions for DNA extraction:

The Bioingenetech Genomic DNA purification Kit contains the following solutions to extract DNA from different samples:

1. Cell Lysis Solution
2. Nuclei Lysis Solution
3. Proteinase K
4. Enzyme-free water
5. Wash Solution
6. Proteinase Precipitation Solution
7. DNA Rehydration Solution

3-1-6: Primers used in polymerase chain reaction:

The primers shown in table (3-5) were used to diagnose *S.aureus* (*nuc*), MRSA (*mecA*) and virulence factors *hlg*, *ebpS*, *fnbB* and *ica A* prepared by Geneaid® (American company).

Table (3-5): The primers used for PCR reaction in the study.

Gene	Primer	sequence	size (bp)	Reference
nuc	*nuc-F*	GCGATTGATGGTGATACGGTT	279	(Rahman *et al.*, 2018)
	nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
mecA	*mecA-F*	GTGAAGATATACCAAGTGATT	147	(Rahman *et al,*, 2018)
	mecA-R	ATGCGCTATAGATTGAAAGGAT		
hlg	*hlg-1*	GCCAATCCGTTATTAGAAAATGC	938	(Jarraud *et al.*, 2002)
	hlg-2	CCATAGACGTAGCAACGGAT		
ebpS	*ebpS-1*	CATCCAGAACCAATCGAAGAC	186	(Peacock *et al.*, 2002)
	ebpS-2	CTTAACAGTTACATCATCATGTTTATCTTTG		
fnbB	*fnbB-1*	GTAACAGCTAATGGTCGAATTGATACT	523	(Tristan *et al.*, 2003)
	fnbB-2	CAAGTTCGATAGGAGTACTATGTTC		
ica A	*ica A-F*	ACACTTGCTGGCGCAGTCAA	188	(Abdrabaa and Aburesha, 2023)
	ica A - R	TCTGGAACCAACATCCAACA		

3-2: Methods:

3-2-1: Preparation of cultural media:

3-2-1-1: Peptone water medium:

Prepare Peptone water medium manufactured by Micro X press®, Malaga/Spain was prepared by dissolving 15 gm of peptone in 1000 ml distilled water and mixing well with heating, and pouring into 8 ml test tubes then autoclaving at 121°C for 15 minutes, allowing to cool at 45 °C.

3-2-1-2: Mannitol salt Agar:

Mannitol Salt Agar medium is a selective medium that facilitates the isolation of *Staphylococcus aureus*.

The medium was prepared according to the manufacturer's instruction (HI-MEDIA®, India) by dissolving 111 grams of the medium in 1 liter of distilled water, then placed on a magnetic stirrer until completely mixed, and autoclaving at 121°C for 15 minutes. After that, the medium was cooled to 45-50°C, then poured into Petri dishes, and stored in the refrigerator for a later use.

3-2-1-3: Blood agar:

Blood agar was used to detect the hemolytic capacity of *S. aureus* by producing different types of hemolytic enzymes, activating the isolated bacteria, and observing hemolysis. It was prepared according to the instructions of the Indian manufacturer, HI-MEDIA®, by dissolving 35g in 1 liter of distilled water, placing it on a magnetic stirrer until boiling, and then autoclaving at 121°C for 15 minutes. After sterilization, the medium was cooled to 45-50°C, and 7% sheep blood was added to it by stirring gently until homogeneous, then poured into sterile Petri dishes and refrigerated until use.

3-2-2: Study Area:

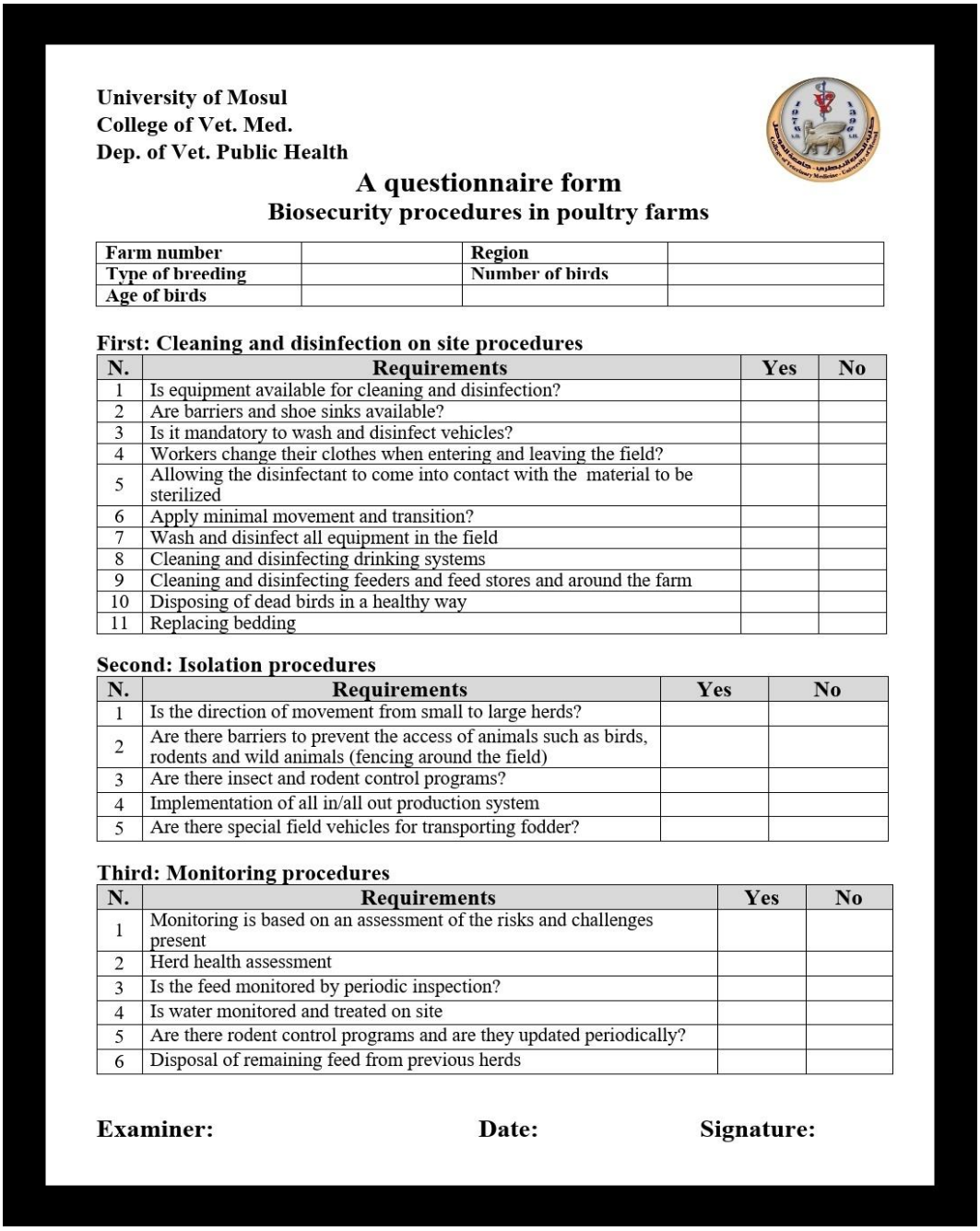
This study was conducted in three Iraqi governorates: Nineveh, Erbil and Duhok, located in the northern part of Iraq between the longitudes 48 and 43 east and the latitudes 36 and 24 north. These governorates are characterized by climatic conditions that vary according to their surface topography, and temperatures range between 0°C - 8°C in winter and between 35°C - 50°C in summer. They are characterized by a great diversity of livestock, as they have many types of domesticated animals such as poultry, sheep, goats, cows and buffaloes, and there are many areas rich in fertile pastures; These governorates are primarily agricultural areas, and animal products constitute the second half of their agricultural production. (Ahmed and Hussein, 2018)

3-2-3: Sampling:

The questionnaire form was adopted through several questions to investigate the application of biosecurity procedures in 26 poultry farms within the different study areas, for three main axes, the first of which was cleaning and disinfection which focused on: (the availability of cleaning and disinfection equipment, availability of barriers and shoe sinks, washing and disinfecting of cleaning vehicles, changing the clothes when entering and leaving the farm, allow the disinfectant to come into contact with the material sterile, applying minimal movement and transition, washing and disinfecting all equipment's in the farm, cleaning and disinfecting drinking systems, cleaning and disinfecting feeders and feed stores and around the farm, disposing of dead birds in a healthy way and replacing bedding).

The second axis was the isolation which focuses on: (the direction of movement from small to large herds, barriers to prevent the access of animals, insect and rodent control programs, implementation of all in/all out production system and special field vehicles for transporting feed).

The third axis was monitoring which focuses on: assessment of the risks and challenges, herd health assessment, feed monitored by periodic inspection, water monitored and treated on-site, rodent control programs and the display of remaining feed from previous herds), which was recorded in a recurring manner among the studied fields, as shown in the Figure (3-1).



University of Mosul
College of Vet. Med.
Dep. of Vet. Public Health

A questionnaire form
Biosecurity procedures in poultry farms

Farm number		**Region**	
Type of breeding		**Number of birds**	
Age of birds			

First: Cleaning and disinfection on site procedures

N.	Requirements	Yes	No
1	Is equipment available for cleaning and disinfection?		
2	Are barriers and shoe sinks available?		
3	Is it mandatory to wash and disinfect vehicles?		
4	Workers change their clothes when entering and leaving the field?		
5	Allowing the disinfectant to come into contact with the material to be sterilized		
6	Apply minimal movement and transition?		
7	Wash and disinfect all equipment in the field		
8	Cleaning and disinfecting drinking systems		
9	Cleaning and disinfecting feeders and feed stores and around the farm		
10	Disposing of dead birds in a healthy way		
11	Replacing bedding		

Second: Isolation procedures

N.	Requirements	Yes	No
1	Is the direction of movement from small to large herds?		
2	Are there barriers to prevent the access of animals such as birds, rodents and wild animals (fencing around the field)		
3	Are there insect and rodent control programs?		
4	Implementation of all in/all out production system		
5	Are there special field vehicles for transporting fodder?		

Third: Monitoring procedures

N.	Requirements	Yes	No
1	Monitoring is based on an assessment of the risks and challenges present		
2	Herd health assessment		
3	Is the feed monitored by periodic inspection?		
4	Is water monitored and treated on site		
5	Are there rodent control programs and are they updated periodically?		
6	Disposal of remaining feed from previous herds		

Examiner: **Date:** **Signature:**

Figure (3-1): The questionnaire form adopted to investigate the application of biosecurity procedures.

This study was conducted on poultry farms distributed in parts of Qandil and Harir areas in Erbil Governorate, Qasrok, Shekhan, Shekhan - mreba, Shekhan - garmishan, Chaman, Barbuhar, Zawita, Amadia, Sarsing, Ashawa, Bagera, Kavilsin and Sarki areas in Duhok Governorate, and Kokjali, Bartella, Tellasquff, Telkaif, Musqlat, Telsin and Teladass areas in Nineveh Governorate during the period from September 2024 to January 2025 as shown in the Figure (3-2), where 234 swabs and samples were collected, including workers hand, chicken, air excreta (ventilatar), feeder, water source, soil, bedding, grass, chicken feed (Table 3-6), where the swabs were placed in tubes containing sterile peptone water, and the samples were placed in special containers prepared for this purpose, and were transferred directly to the Scientific Research Laboratory at the College of Veterinary Medicine at the University of Mosul for Necessary bacteriological laboratory tests.

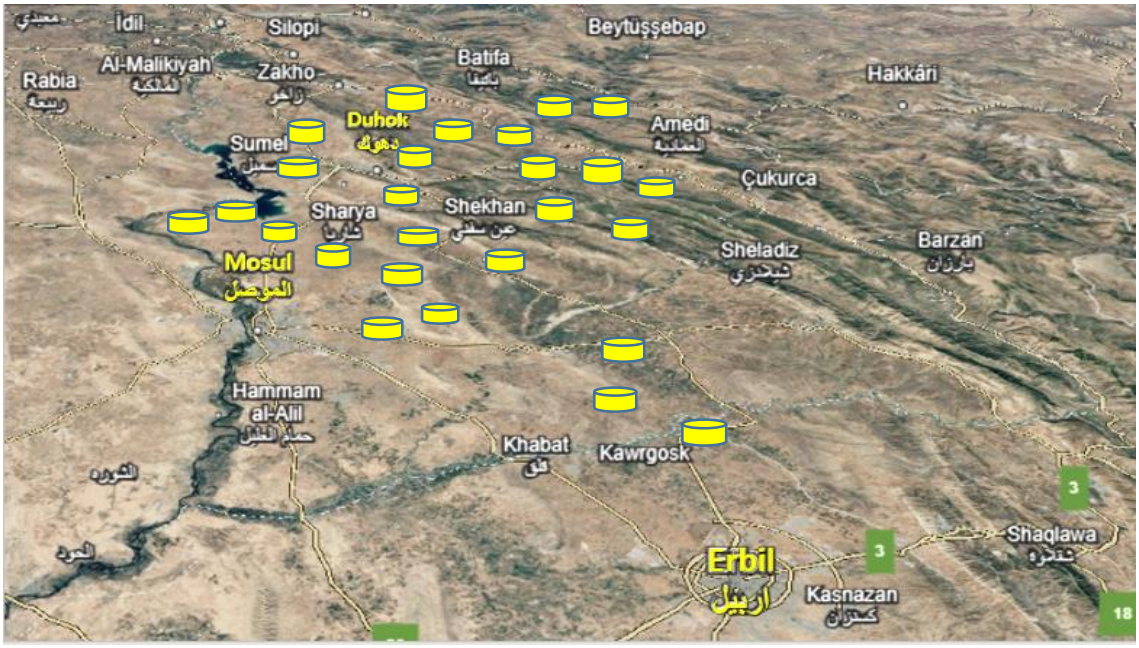


Figure (3-2): Geographical distribution of the collected samples.

Table (3-6): Total samples according to their type.

No.	Type of sample	No. of Samples
1	Workers hand	26
2	Chicken	26
3	Ventilator	26
4	Feeder	26
5	Water	26
6	Soil	26
7	Bedding	26
8	Grass	26
9	Chicken feed	26
Total number of samples		**234**

3-2-4: Data collection and management:

The questionnaire was pre-selected to ensure that all important issues were identified and covered. and Data were collected through field visits to poultry farms conducting a face-to-face interview with farm owners, in addition, to observing and verifying these procedures. The answers to the questionnaire were documented as yes or no on biosecurity procedures and then compared to the ideal biosecurity standard (Al-Mahmood, 2023).

Data were entered into a Microsoft Excel program designed to retain, store and retrieve field survey data and results during analysis. The percentages of answers were calculated by dividing the number of yes or no / number of criteria adopted in the questionnaire multiplied by 100.

3-2-5: Sample preparation and bacterial isolation:

3-2-5-1: Swab samples:

The swabs of worker's hand, chicken, ventilator and feeder were placed in a peptone water and then incubated at 37°C for 2-3 hours, then transferred to Mannitol Salt Agar and incubated at 37°C for 24-48 hours. The results were read based on the cultural characteristics of the bacterial

colonies, such as the shape, texture, size and color of the colonies, mannitol fermentation change in the color of the medium from red to yellow and the growth of golden-yellow colonies (Koneman *et al.,* 1997b).

3-2-5-2: Water samples:

According to MacFaddin (2000) method was adopted to isolate *S. aureus* bacteria from water by taking 10 ml of water sample and placing it in a centrifuge at 4000 rpm for 15 minutes. After that, the supernatant was discarded and 0.2 ml of the sediment was transferred by pipette to Mannitol Salt Agar medium and incubated at 37°C for 24-48 hours. Then, the results were read based on the cultural characteristics of the bacterial colonies such as the shape, texture, size and color of the colonies, mannitol fermentation, change in the color of the medium from red to yellow, and the growth of golden-yellow colonies.

3-2-5-3: Soil, bedding, grass and chicken feed samples:

According to Burriel (1997) method was used to isolate bacteria from soil, bedding, grass and chicken feed samples by placing 10 g of sample in sterile flasks containing 90 ml of sterile phosphate-buffered saline (PBS) solution and shaking well for one minute and leaving it for two hours to separate the bacteria attached to the samples. Then the liquid was distributed in sterile test tubes and placed in a centrifuge at 5000 rpm for 5 minutes. After that, 100 microliters of the supernatant were taken and transferred to Mannitol Salt Agar medium and incubated at a temperature of 37 °C for 24 to 48 hours. The results were read based on the cultural characteristics as mentioned previously.

3-2-6: *Staphylococcus aureus* Identification:

Golden-yellow colonies typically appear on mannitol salt agar, changing the medium color from red to yellow due to mannitol fermentation, and several isolations may be required to obtain purified isolates (Koneman *et al.*,1997a)

3-2-7: Microscopical examination:

Smears were made from the positive colonies growing on Mannitol Salt Agar medium and stained with Gram stain. Their shape, size, arrangement, and staining reaction were noted for the purpose of diagnosing staphylococci, which under the microscope appear as, diploid cells resembling grape clusters, and are positive for gram stain.

3-2-8: Biochemical tests:

3-2-8-1: Catalyse test:

Part of the growing colony was taken by a loop and placed on a glass slide, then a drop of 3% hydrogen peroxide H_2O_2 was added and mixed. The positive result was observed by the formation of bubbles, as the catalase enzyme breaks down hydrogen peroxide and produces free oxygen, which is released in the form of bubbles (Koneman *et al.*, 1997a; MIDI, 2004).

$$H_2O_2 + \text{catalase} = H_2O + 1/2\ O_2$$

3-2-8-2: Coagulase test:

The test was performed by taking a part of the colony of the isolated bacteria and mixing it with a drop of physiological saline solution, on a clean glass slide, then a drop of sheep plasma was added to this mixture, and mixed well on the slide. A positive result was observed by the coagulation reaction that occurs on the slide after 1-2 minutes (Isenberg, 1992; Koneman *et al.*, 1997a).

3-2-9: Molecular diagnosis using Polymerase Chain Reaction (PCR):

3-2-9-1: DNA Extraction:

DNA of *S. aureus* was extracted using the Bacterial DNA Preparation Kit as follows:

1- Newly grown colonies were transferred from agar medium to 2 ml Eppendorf tubes containing cell lysis solution and mixed well using Vortex. The sample was placed in a centrifuge for 30 seconds at 13000 rpm.

2- Five hundred microliters of Lysozyme solution 50mg/ml were added to the sample and mixed well using vortex.

3- The solution was incubated at 37°C for 60 minutes in a water bath.

4- The sample was placed in a centrifuge at 13000 rpm for 3 minutes, discarding the supernatant.

5- 200 µl of both lysis and 20 µl of Proteinase K solutions 20mg/ml were added and mixed well.

6- The sample was incubated at 56°C in a water bath for 60 minutes.

7- 200 µl of a binding solution and 200 µl of an absolute ethanol were added and mixed well for 15 seconds.

8- The tubes were placed in an Eppendorf microcentrifuge, then centrifuged at 15000 rpm for three minutes.

9-500 to 600 µl of the samples were transferred to a spin column and placed in a centrifuge at 13000 rpm for a single minute.

10-500 µl of the first wash solution was added to the spin column using the collection tube and centrifuged at 13000 rpm for 1 min; then filtered the liquid through it and collected the spin column in the 2 ml collection tube.

11-500 µl of the second wash solution was added to the spin column using the collection tube and centrifuged at 13000 rpm for 1 min, then filtered

the liquid through it and collected the spin column using the 2 ml collection tube.

12- Additional centrifugation at 13000 rpm for 1 min to remove the remaining ethanol in the spin column.

13-The spin column was then transferred to a new 1.5 ml microcentrifuge tube and 100 µl of elution solution was added to the spin column and left for 1 min.

16- Genomic DNA was recovered by centrifugation at 13000 rpm for one minute.

17- Finally, the extracted DNA was stored in a freezer at -20°C until further use.

3-2-9-2: Gel electrophoresis

To perform DNA electrophoresis, 1% agarose gel was prepared by dissolving 0.5 g of agarose powder in 50 ml of (Tris-boric acid EDTA) 1X TBE and adding 3 µl of Safe Red dye using a heat source with continuous stirring until boiling, then let it cool to a temperature of (50-60) degrees Celsius. The gel was poured into the electrophoresis device tray after installing the special comb to form wells on the edges of the gel, carefully pouring to avoid bubble formation.

Subsequently, the tray is placed in the electrophoresis basin containing an appropriate amount of 1X TBE solution. Samples were prepared by mixing (5) microliters of the DNA sample with (3) microliters of the loading solution. After that, the relay device was operated by passing an electric current with a voltage difference of (5) volts/cm, and the process takes (60-70) minutes.Lastly, the gel was photographed under ultraviolet rays using UV transillumination gel imaging to see the DNA bands and also the result of the PCR reaction.

3-2-9-3: PCR reactions

The DNA concentrations in all study samples were adjusted by dilution with (Tris EDTA) TE buffer solution to obtain the required concentration for PCR reactions, which was 50 ng/microliter for each sample.The master mixture reaction was prepared for each PCR polymerase reaction by mixing the DNA sample (template) and the primers specific to each gene with the master-mix components inside an Eppendorf tube with a capacity of 0.2 ml. The reaction volume was fixed to 20 microliters with nuclease-free water. The mixture was then vortexed for (3-5) seconds to ensure that the reaction components were mixed well.

The reaction tubes were inserted into the thermocycler for the purpose of performing the amplification using the specific program for each reaction (Tables 3-7, 3-8 and 3-9).

Table (3-7): PCR reaction program for *nuc* and *mec A* gene:

No.	Steps	Temperature °C	Time min	No. of cycles
1	Initial denaturation	95	10	1
2	Denaturation	95	1	35
3	Annealing	55 nuc 60 mec *A*	1	
4	Extension	72	1	
5	Final Extension	72	5	1

Table (3-8): PCR reaction program for *hlg*, *ebpS* and *fnbB* genes:

No.	Steps	Temperature °C	Time min	No. of cycles
1	Initial denaturation	95	10	1
2	Denaturation	95	1	35
3	Annealing	55	1	
4	Extension	72	1	
5	Final Extension	72	5	1

Table (3-9): PCR reaction program for *ica A* gene:

No.	Steps	Temperature °C	Time min	No. of cycles
1	Initial denaturation	95	10	1
2	Denaturation	95	1	35
3	Annealing	60	1	
4	Extension	72	1	
5	Final Extension	72	5	1

After that, the sample was loaded into the wells of the agarose gel prepared in advance at a concentration of 1% with the addition of the DNA ladder.

Finally, the samples were transferred by running the electrophoresis for a period ranging between (60-70) minutes.

3-2-10: Statistical Analysis:

Pearson correlation test was used to find the relationship between the percentages of the applied biosecurity measures, the age of birds studied and the percentage of isolation obtained from the different samples in the different geographic study sites at $P < 0.05$ (Thrane, 2024).

Chapter Four

Results

4-1: The isolation and Identification of *S. aureus* by traditional and molecular methods

The isolation results of *S. aureus* showed that 101 samples out of 234 samples were positive, i.e. 43.16% of the total samples included in the study (workers' hands, chicken, ventilator, feeder and water, soil, bedding, grass and chicken feed). The highest isolation rate was from workers' hand and chicken to record the percentage 53.85%, and the lowest isolation rate was from Feeder and Water to record the percentage 34.61%, as shown in Table (4-1).

Table (4-1): Number and percentage of *S. aureus* isolates from different samples

No.	Sample type	No. of Samples	No. of +Ve Samples	%
1	Workers hand	26	14	53.85
2	Chicken	26	14	53.85
3	Ventilator	26	12	46.15
4	Feeder	26	9	34.61
5	Water	26	9	34.61
6	Soil	26	12	46.15
7	Bedding	26	10	38.46
8	Grass	26	11	42.31
9	Chicken feed	26	10	38.46
Total		**234**	**101**	**43.16**

The highest prevalence rate of *S. aureus* was recorded among samples from different fields and from different geographical sites from the workers' hands and chickens at 13.86%, while the lowest prevalence rate was in the feeder and water samples at 8.91%, as shown in Table (4-2) and figure (4-1).

Table (4-2): Prevalence of *S. aureus* of the different studied samples.

No.	Type of sample	No. of Samples	No. of +Ve Samples	%
1	Workers hand	26	14	13.86
2	Chicken	26	14	13.86
3	Ventilator	26	12	11.88
4	Feeder	26	9	8.91
5	Water	26	9	8.91
6	Soil	26	12	11.88
7	Bedding	26	10	9.9
8	Grass	26	11	10.89
9	Chicken feed	26	10	9.9
Total		**234**	**101**	**100**

The results showed that the highest isolation rate was in Erbil Governorate at 60.32%, followed by Nineveh Governorate at 44.44%, and a lowest rate was in Duhok Governorate at 35.42% of the total samples included in the study, as shown in Table (4-3).

Table (4-3): Number and percentage of *S. aureus* isolates from different regions.

No.	Region	No. of Farms	No. of Samples	No. of +Ve Samples	%
1	Duhok	16	144	51	35.42
2	Erbil	3	63	38	60.32
3	Nineveh	7	27	12	44.44
Total		**26**	**234**	**101**	**43.16**

The highest prevalence rate was recorded in Duhok Governorate, followed by Erbil Governorate and then Nineveh Governorate, with rates of 50.50%, 37.62% and 11.88%, respectively, as shown in Table (4-4) and Figure (4-2).

Table (4-4): Prevalence of *S. aureus* in different studied geographical regions.

No.	Region	No. of Farms	No. of Samples	No. of +Ve Samples	%
1	Duhok	16	144	51	50.50
2	Erbil	3	63	38	37.62
3	Nineveh	7	27	12	11.88
Total		**26**	**234**	**101**	**100**

The results of the initial isolation by traditional methods of *S. aureus* on MSA showed the growth of medium to large, round, smooth, soft, elevated and shiny yellow colonies with a change in the color of the medium from red to yellow (Figure 4-1).

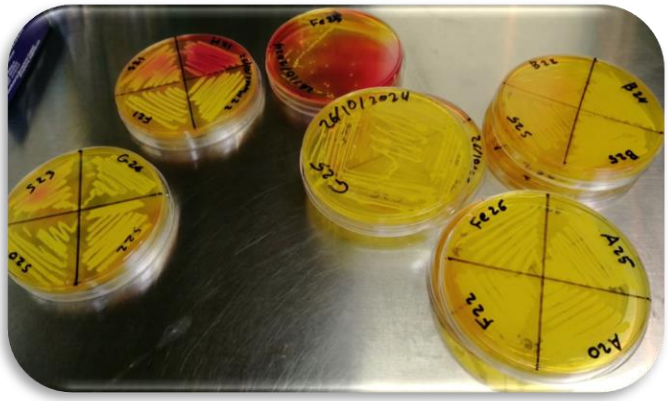


Figure (4-1): Growth of *S. aureus* colonies on MSA.

Microscopically, the bacterial colonies were Gram-positive and grape-shaped, arranged in single cells or pairs, or forming irregular coccoid groups resembling grape clusters (Figure 4-2).

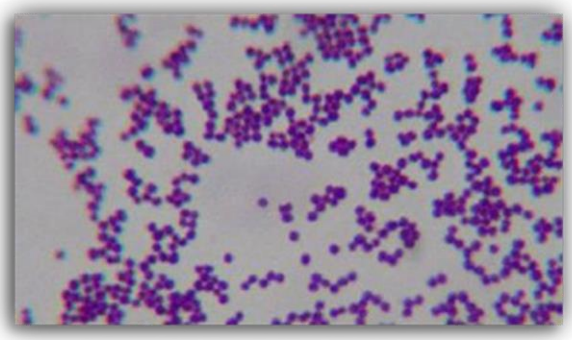


Figure (4-2): The microscopic image of *S. aureus* (100X).

On blood agar, resulted in beta hemolysis, clear, transparent zone around the colonies due to complete lysis of red blood cells (Figure 4-3).

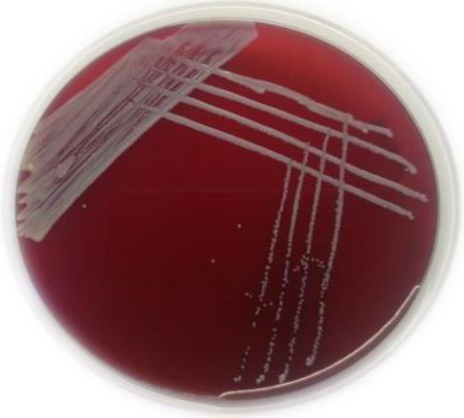


Figure (4-3): Growth of *S. aureus* colonies on blood agar.

Biochemically, the isolates showed positive results for the catalase reagent test, because of the ability to convert hydrogen peroxide (H_2O_2) into water and oxygen gas, as the positive result appeared in the form of gas bubbles, as shown in Figure (4-4).

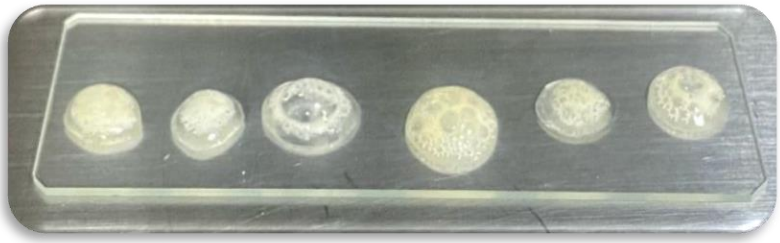


Figure (4-4): Slide catalase test.

The coagulase test is a unique test used to confirm *S. aureus* infection. *S. aureus* isolates had shown positive results in the plasma coagulase test, as shown in Figure (4-5).

Figure (4-5): Slide coagulase test.

Molecularly, the results of the amplified DNA extract of *S. aureus* on agarose gel showed that matching of DNA bands with marker size of *nuc* gene region at 279 bp, as a specific for the diagnosis of *S. aureus as* shown in Figure (4-6).

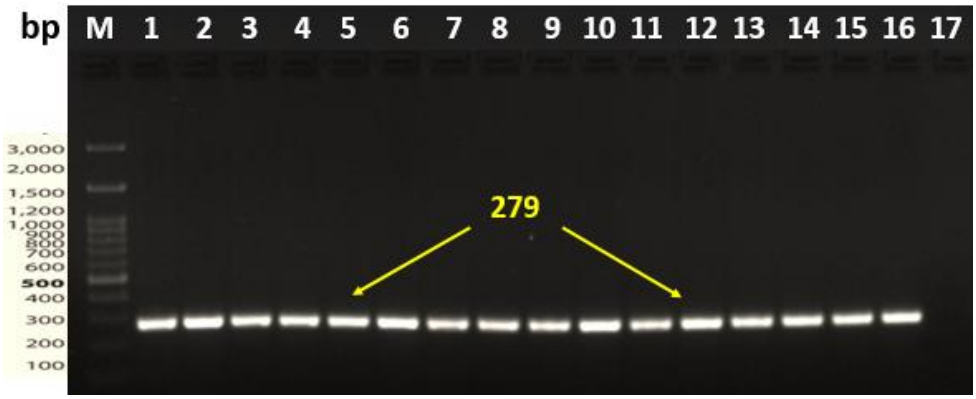


Figure (4-6): PCR reaction product for *S. aureus* isolates for *nuc* gene at 279 bp.

4-2: Results of the questionnaire to investigate the biosecurity procedures:

The results of the questionnaire were included in order to investigate the biosecurity procedures for 22 categories for 26 fields, which include: (availability of cleaning and disinfection equipment, availability of barriers and shoe sinks, washing and disinfecting cleaning vehicles, changing the clothes when entering and leaving the farm, allowing the disinfectant to come into contact with the material to be sterilized, applying minimal movement and transition, washing and disinfecting all equipment's in the farm, cleaning and disinfecting drinking systems, cleaning and disinfecting feeders and feed stores and around the farm, disposing of dead birds in a healthy way, replacing bedding, direction of movement from small to large herds, barriers to prevent the access of animals, insect and rodent control programs, implementation of all in/all out production system, and special field vehicles for transporting feed, Monitoring is based on an assessment of the risks and challenges, herd health assessment, feed monitored by periodic inspection, water monitored and treated on site, rodent control programs and disposal of remaining feed from previous herds), which was recorded in a recurring manner among the studied fields. The application

of 19, i.e. 86.36% of the categories was documented in field No. 6 at the age of 7 days, in which no isolation rate was recorded. On the contrary, in field No. 22, which was 40 days old, the application of 5 categories was documented representing 22.73%, and recorded an isolation rate of 100% in the studied samples.

The age of the chicken flocks also had an impact on the application of biosecurity measures and the isolation rates. The older the flock, the less biosecurity measures were applied, which was reflected in the isolation rate (Table 4-5).

Table (4-5): Percentages of applied biosecurity measures versus bacterial isolation rates and ages in the studied farms from different areas covered by the study.

Field No.	Biosecurity procedures			*S. aureus* isolates from different sources		Age (day)
	No. of studied categories	No. of applied categories (%)	Not application No.(%)	No. of samples	No. of +ve samples (%)	
1	22	6(27.27)	16(72.73)	9	7(77.77)	36
2	22	11(50.00)	11(50.00)	9	7(77.77)	36
3	22	18(81.81)	4(18.19)	9	2(22.22)	15
4	22	18(81.81)	4(18.19)	9	2(22.22)	18
5	22	18(81.81)	4(18.19)	9	1(11.11)	11
6	**22**	**19(86.36)**	**3(13.64)**	**9**	**0(00.00)**	**7**
7	22	15(68.18)	7(31.82)	9	3(33.33)	28
8	22	22(100.00)	0(00.00)	9	1(11.11)	15
9	22	20(90.90)	2(10.10)	9	4(44.44)	32
10	22	20(90.90)	2(10.10)	9	1(11.11)	15
11	22	18(81.81)	4(18.19)	9	2(22.22)	22
12	22	14(63.64)	8(36.36)	9	5(55.55)	34
13	22	11(50.00)	11(50.00)	9	2(22.22)	22
14	22	9(40.91)	13(59.09)	9	2(22.22)	23
15	22	13(59.10)	9(40.90)	9	5(55.55)	35
16	22	15(68.18)	7(31.82)	9	4(44.44)	33
17	22	8(36.36)	14(63.64)	9	7(77.77)	35
18	22	12(54.55)	10(45.45)	9	3(33.33)	25
19	22	10(45.45)	12(54.54)	9	4(44.44)	32
20	22	10(45.45)	12(54.54)	9	4(44.44)	28
21	22	8(36.36)	14(63.64)	9	5(55.55)	34
22	**22**	**5(22.73)**	**17(77.72)**	**9**	**9(100)**	**40**
23	22	15(68.18)	7(31.82)	9	6(66.66)	36
24	22	8(36.36)	14(63.64)	9	4(44.44)	35
25	22	5(22.73)	17(77.27)	9	7(77.77)	35
26	22	13(59.10)	9(40.90)	9	1(11.11)	15

The direction of movement from small to large herds procedure was the least applied biosecurity procedure, as it was applied in 3% of the studied fields. On the contrary, the categories procedure implementation of all in/all out production system was the most applied biosecurity procedure in poultry farms, as it was applied in 100% of the studied fields, it is believed that it is one of the contributing factors in the occurrence of contamination, as shown in table (4-6).

Table (4-6): Percentages of application of each category of biosecurity in the studied farms from different geographical areas include in the study.

Category No.	Type of biosecurity category	No. of farms	No. of applied farms (%)	No. of not applied farms (%)
1	Availability of cleaning and disinfection equipment	26	14(53.85)	12(46.15)
2	Availability of barriers and shoe sinks	26	14(53.85)	12(46.15)
3	Wash and disinfect vehicles	26	10(38.46)	16(61.54)
4	Changing the clothes when entering and leaving the farm	26	12(46.15)	14(53.85)
5	Allowing the disinfectant to come into contact with the material to be sterilized	26	7(26.92)	19(73.08)
6	Apply minimal movement and transition	26	14(53.85)	12(46.15)
7	Wash and disinfect all equipments in the farm	26	17(65.38)	9(34.62)
8	Cleaning and disinfecting drinking systems	26	17(65.38)	9(34.62)
9	Cleaning and disinfecting feeders and feed stores and around the farm	26	15(57.69)	11(42.31)
10	Disposing of dead birds in a healthy ways	26	19(73.08)	7(26.92)

11	Replacing bedding	26	25(96.15)	1(3.85)
12	**Direction of movement from small to large herds**	**26**	**3(11.54)**	**23(88.46)**
13	Barriers to prevent the access of animals	26	13(50.00)	13(50.00)
14	Insect and rodent control programs	26	19(73.08)	7(26.92)
15	**Implementation of all in/all out production system**	**26**	**26(100.00)**	**0(00.00)**
16	Special field vehicles for transporting feed	26	24(92.31)	2(7.69)
17	Monitoring is based on an assessment of the risks and challenges	26	20(76.92)	6(23.08)
18	Herd health assessment	26	18(69.23)	8(30.77)
19	Feed monitored by periodic inspection	26	5(19.23)	21(80.77)
20	Water monitored and treated on site	26	7(26.92)	19(73.08)
21	Rodent control programs	26	17(65.38)	9(34.62)
22	Disposal of remaining feed from previous herds	26	25(96.15)	1(3.85)

4-3: The relationship between isolation rates and the application of biosecurity measures:

The data obtained from the Pearson correlation test found the inverse relationship between the application of biosecurity measures and the isolation rates of *Staphylococcus aureus* in the areas covered by the study, as the relationship shows an increase in pollution rates with a decrease in the application of biosecurity measures as shown in Figure (4-7).

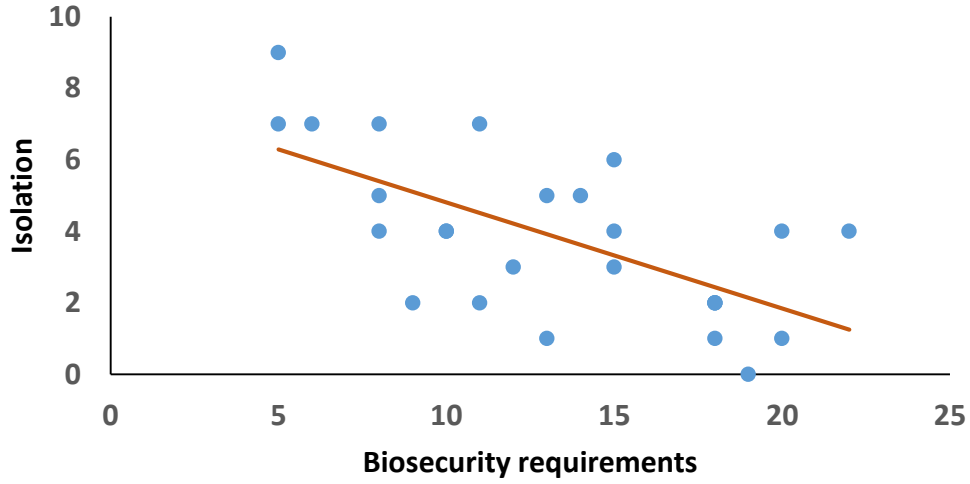


Figure (4-7): Correlation between the applied biosecurity measures and *S. aureus* isolation among different samples in studied farms from different areas covered by the study. Pearson's Correlation (r = -0.6425, P-value < 0.01).

The same effect was found between the isolation rate of *S. aureus* and the age of the birds at (P < 0.01), as shown in Figure (4-8) and Table (4-5).

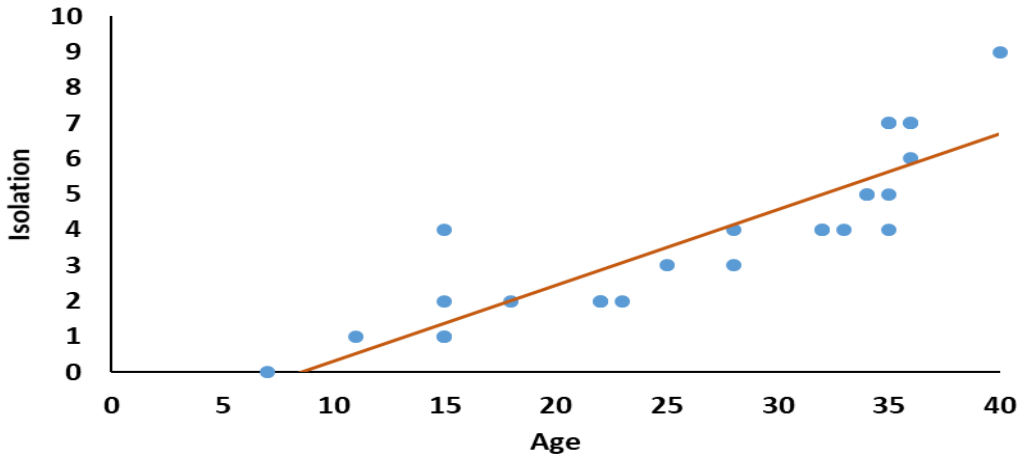


Figure (4-8): The correlation between *S. aureus* isolation and the birds age in the studied farms from different areas covered by the study. Pearson's Correlation (r = 0.8766, P-value < 0.01).

4-4: Isolation and identification of MRSA:

The results of MRSA isolation showed that (38) samples out of (234) samples were positive, *i.e.* 16.24% by culturing chromogenic agar medium and molecular methods by electrophoresis of amplification products for *mecA* gene at 147 bp on an agarose gel (Figures 4-11 and 4-12). The highest percentages of MRSA isolation were recorded from bedding and chicken at 34.61% and 26.92%, respectively, and the lowest percentages of isolation were from grass, soil and chicken feed at 3.85%, 7.69% and 7.69%, respectively (Table 4-7).

Table (4-7): Numbers and percentages of MRSA isolates from different samples.

No.	Type of sample	No. of Samples	No. of +Ve Samples	%
1	Workers hand	26	5	19.23
2	Chicken	26	7	26.92
3	Ventilator	26	3	11.53
4	Feeder	26	5	19.23
5	Water	26	4	15.38
6	Soil	26	2	7.69
7	Bedding	26	9	34.61
8	Grass	26	1	3.85
9	Chicken feed	26	2	7.69
Total		**234**	**38**	**16.24**

The results of MRSA isolation revealed a match between the isolation results on chromogenic agar, which is bluish-green in color, and the identification by molecular methods (Figures 4-9 and 4-10).

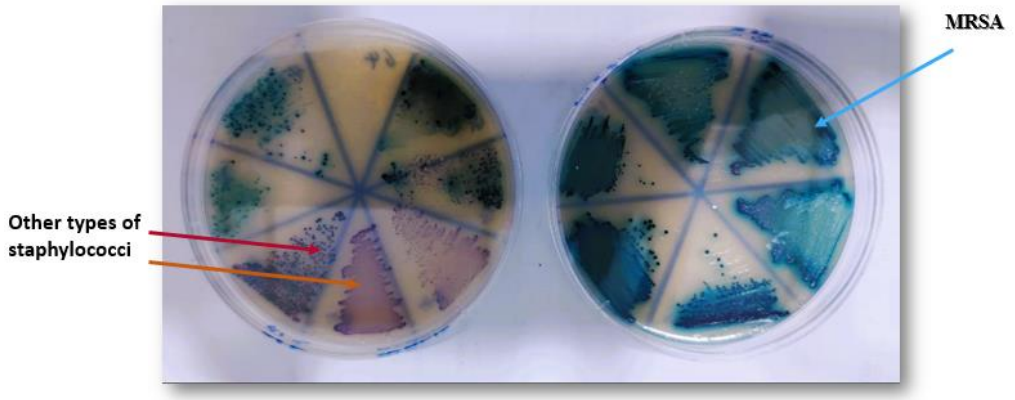


Figure (4-9): The growth of methicillin-resistant *S.aureus* (MRSA) on MRSA chromogenic agar, appeared bluish-green in color.

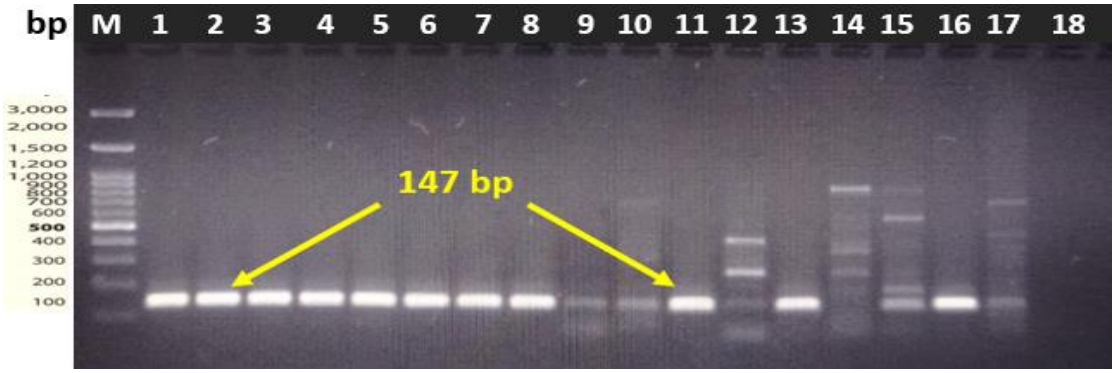


Figure (4-10): PCR reaction product of MRSA isolates for the *mecA* gene at 147 bp.

The highest prevalence rate of MRSA was recorded among the samples from different fields and from different areas, from the bedding and chicken at 23.68% and 18.42%, respectively, while the lowest prevalence rates were in the grass, soil and chicken feed samples at 2.63%, 5.26% and 5.26%, respectively, as shown in Table (4-8).

Table (4-8): Prevalence of MRSA of different studied samples

No.	Type of sample	No. of Samples	No. of +Ve Samples	%
1	Workers hand	26	5	13.16
2	Chicken	26	7	18.42
3	Ventilator	26	3	7.89
4	Feeder	26	5	13.16
5	Water	26	4	10.53
6	Soil	26	2	5.26
7	Bedding	26	9	23.68
8	Grass	26	1	2.63
9	Chicken feed	26	2	5.26
Total		**234**	**38**	

4-5: Detection of virulence genes for MRSA:

The study also revealed the presence of virulence genes for MRSA, which were evident through the results of amplification and electrophoresis on agarose gel by appearance of bands of *fnbB* gene at product size of 523 bp and 188 bp for *ica A* gene and 938 bp for *hlg* and 186 bp for *ebp S* gene at rates of 63.2%, 57.9%, 60.5% and 50%, respectively, out of 38 isolates from different sources.

The *fnbB* gene was detected in MRSA isolates from chicken, ventilator, feeder, soil, bedding, grass and chicken feed at the rates of 100%, 67%, 80%, 50%, 89%, 100% and 50%, respectively, *ica A* gene was found in the workers hand, chicken, ventilator, soil, bedding, and chicken feed isolates at the rates 80%, 86%, 67%, 100%, 67%, and 100%, respectively, *hlg* gene was recorded in MRSA isolates from workers hand,

ventilator, feeder, water, soil, bedding, and chicken feed at 60%, 100%, 60%, 100%, 50%, 78%, and 100%, respectively, while the *ebpS* gene was detected in MRSA isolates from workers hand, chicken, ventilator, feeder, water, bedding, grass, and chicken feed at 80%, 86%, 67%, 20%, 50%, 11%, 100%, and 100%, respectively as shown in Table (4-9), Figures (4-11) and (4-12).

Table (4-9): Numbers and percentages of virulence genes of MRSA isolates from different sources.

No.	**Source**	**No. of Isolate**	**Virulence genes**							
			Fnb B		***ica A***		***hlg***		***ebp S***	
			No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
1	Workers hand	5	0	0	4	80	3	60	4	80
2	Chicken	7	7	100	6	86	0	0	6	86
3	Ventilator	3	2	67	2	67	3	100	2	67
4	Feeder	5	4	80	0	0	3	60	1	20
5	Water	4	0	0	0	0	4	100	2	50
6	Soil	2	1	50	2	100	1	50	0	0
7	Bedding	9	8	89	6	67	7	78	1	11
8	Grass	1	1	100	0	0	0	0	1	100
9	Chicken feed	2	1	50	2	100	2	100	2	100
Total		38	24	63.2	22	57.9	23	60.5	19	50

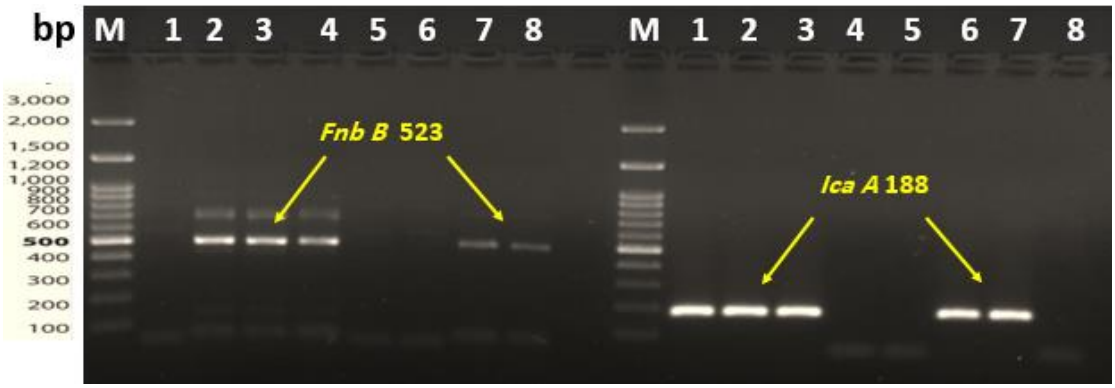


Figure (4-11): PCR reaction product of *S. aureus* isolates for the *fnbB* gene at 523 bp and *ica A* gene at 188 bp.

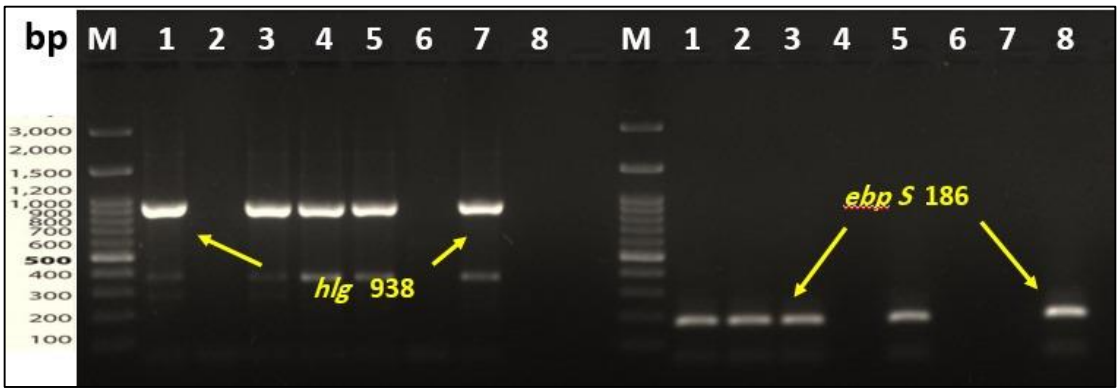


Figure (4-12): PCR reaction product of *S. aureus* isolates for the *hlg* gene at 938 bp and *ebpS* gene at 186 bp.

The frequency of virulence genes varied in different isolates, where the highest frequency of *fnbB, ica A, hlg* and *ebp S* genes was recorded in 37% of the isolates from ventilator, bedding and chicken feed, and the lowest frequency of the *fnbB* and *ebp S* genes was recorded in grass isolates at 3%, as shown in Table (4-10).

Table (4-10): Frequency rates of *fnbB, ica A, hlg* and *ebp S* genes in MRSA isolates.

No.	**Virulence gene**	**Frequency**	
		No. of Isolates	**%**
1	*fnbB + ica A + hlg + ebp S*	14	37
2	*fnbB + hlg + ebp S*	5	13
3	*fnbB + ica A + hlg*	2	5
4	*fnbB + ica A + ebp S*	7	18
5	*ica A + hlg + ebp S*	5	13
6	*fnbB + ebp S*	1	3
7	*hlg + ebp S*	4	11
Total		38	100

Chapter Five

Discussion

The poultry industry in Iraq is one of the country's most important agricultural sectors. With a continued population growth and increased demand for poultry meat, the poultry sector in Iraq is expected to continue to develop and grow in the future. Several factors play a role in improving the poultry industry in Iraq and enhancing its future. These include increased domestic demand due to population growth, improved income, changing lifestyles, and increased nutritional awareness among the population, as well as increased investment in technological upgrades and infrastructure development. In short, the future of the poultry industry in Iraq is expected to experience continued economic growth motivated by increased domestic demand, technological improvements, and investments. The transition from primitive to industrial farms and products diversification have also played a significant role in improving efficiency, quality, and competitiveness. Despite all these positive factors, the industry also faces challenges, such as market fluctuations, rising production costs, security instability in some areas, and disease outbreaks. (Mohammadali Tabar *et al*,2024)

The poultry industry is an important sector that meets the demand for protein with quick, financial returns and easy to marketing, provided that good feed is available and certain preventative measures are implemented (Hafez *et al.,* 2022). Poultry farming systems in Iraq are classified as closed systems and rely on floor-based farming (Kshash and Oda, 2019).

A critical aspect of poultry farming, both at the health and administrative levels, is biosecurity, which is an integral part of any type of animal production (Kustritz, 2022). The Food and Agriculture

Organization recommends the strict implementation of biosecurity programs and measures as the most effective means of disease prevention and control (FAO, 2020).

One of the most widely used and common methods for assessing biosecurity practices and procedures in poultry production is the detection and measurement of pathogenic bacteria as an indicator of hygiene, such as *S. aureus*, which is commensal with poultry environment, which is the pioneer bacterial agent in communities worldwide (Santos *et al.*, 2020). Its importance as a pathogen in animals and humans is worth highlighting, given its ability to cause infection, its adaptability to diverse environmental conditions, and its resistance to multiple antimicrobials (WHO 2017; Santos *et al.*, 2020).

In northern Iraq, we have not observed any studies or data on linking between biosecurity applications and the levels of contamination with microorganisms, especially *S. aureus*, in poultry farms and their surrounding environment. Therefore, it is decided to study this topic because of its great importance in both poultry industry and human health.

In this study, *S. aureus* was used as an indicator of the success and failure of biosecurity measures in poultry farms. Through the questionnaire that was conducted on the studied farms, it was found that there was a disparity in the application of biosecurity measures in different fields, which cast a shadow on the isolation and spread rates of *S. aureus* from different sources in the poultry farms under study. There was a clear inverse relationship between the low isolation rate and the high biosecurity application index, and this was confirmed by Hafez *et al.* (2022).

The study revealed a difference in the application of biosecurity requirements in the poultry farms covered by the study. The reasons for that were due to the unwillingness of breeders to apply some requirements or procedures as they believe that they may add a financial burden to them

under the fluctuations in the poultry markets. The other reason was also due to the lack of application or the failure of application as the birds age. The older birds in the poultry farms, the less to be found in the application of biosecurity procedures or requirements or their lack of strict application. This was clear from the results obtained. In reviewing to Table No. (4-5), it was found that the isolation rate in Farm No. 6 at the age of 7 days was 0%, as 19 requirements were applied out of a total of 22 requirements, *i.e*. 86.36% of the biosecurity requirements, which are considered good biosecurity. On the contrary, in Farm No. 22, the isolation rate was 100%, while applying 5 requirements out of a total of 22, *i.e.* 22.73% of what was required, which was considered a bad biosecurity score. That would lead to air pollution, which would be considered a reservoir for microscopic organisms and led to pollution. Farm components with pathogens, including *S. aureus*, cast their shadows on the health and productivity of poultry due to the increasing concentrations of their numbers with age, that was what Vučemilo *et al.* (2010) indicated.

S. aureus is known as a pathogen capable of invading the human and animal body and is responsible for the occurrence of various diseases and infections (Schaumburg *et al.*, 2015). Although the results of isolation and diagnosis of *S. aureus* were identical using traditional and molecular methods in our study, the main reliance was on the results of diagnosis and characterization using molecular methods to obtain the best and most accurate results, as they are more sensitive, as indicated by (Phuektes *et al.*, 2001). The result showed that there were 101 samples out of 234 samples were positive, *i.e.* 43.16% of the total samples which included workers' hands, chicken skin, ventilator, feeder, water, soil, bedding, grass and chicken feed, the highest rate and prevalence were samples of workers hands and chickens.

The isolation rate of S. aureus in our study was higher than the results recorded by Almousawi and Alhatami (2020) in Babylon, which amounted to 37.7%, and the results submitted by Abdulrahman (2020) in Duhok, which amounted to 28%.

In another study similar to ours, conducted on six poultry farms in Ethiopia on chickens, workers, bedding and water to investigate *S. aureus*, the results were lower than our results, with the rates being 17%, 15%, 4.7% and 7%, respectively (Abunna *et al*., 2020).

It was lower than the prevalence rate recorded in the study conducted by Pondit *et al.* (2018), which was conducted in Bangladesh on chickens and quails, where the prevalence rate of 10.45% for chickens was recorded based on the *nuc* gene.

The present study showed that out of a total of 234 samples, 38 samples, or 16.24%, were positive for MRSA from all samples included in the study and 19.23% from workers' hand and 26.92% chicken. Compared to studies conducted on workers and poultry, the study conducted by Assafi *et al.* (2020) in Duhok province recorded isolation rates of 24% and 27.3%, respectively, which were similar between the rates we reached in the current study.

In comparison with the studies conducted locally by AL-Salihi *et al.* (2023), a study conducted in Kirkuk Governorate on poultry workers recorded a rate of 16.6%, which was less than the rate reached, which was 19.23%. The reason for the difference was likely to be due to the difference in the sample collection area, as the first was from the nose and the second was from the hands. The results were largely similar to the ones reached by Hado and Assafi, (2021) in Duhok Governorate, which reached 27.77% in chicken when compared to the present results, which reached 26.92%. While the isolation results of MRSA from poultry meat varied between

33.3%, 40%, and 14.81% in Wasit, Iran, and Basra (Kanaan, 2018; Jafarzadeh *et al.*, 2023; Aziz *et al.*, 2024).

Internationally, studies had recorded varying isolation rates of MRSA from poultry, including 43.3% in Korea (Suk et al., 2010), 71.5% in Germany (Richter et al., 2012), 36% in Egypt (Karmi, 2013), 67% in Iran (Rahimi and Karimi, 2015), and 52% in Denmark (Tang et al., 2017).

No observance for any studies that addressed the isolation and diagnosis of MRSA from ventilator, feeder, water, soil, bedding, grass and chicken feed in poultry farms, despite their importance in the occurrence of transmission and contamination (Abd El-Ghany, 2021). However, in an epidemiological study conducted by Hussein *et al.* (2015) to investigate the presence of bacteria in the air inside poultry farms, it was recorded at a rate of 50%, which was a major cause of water and food contamination for workers, etc.

The variation in the isolation rates obtained in this study compared to other studies may be explained by many factors, including the administrative and health practices of poultry farms, breeding methods, the diagnostic methods used, geographical differences, etc., in addition to other factors like the environment in which poultry are raised, such as water, soil and bedding, feces and waste, sick and dead birds, and other poultry products, that was what was confirmed by Khan *et al.* (2014) and Laban *et al.* (2014).

The possession of the *mecA* gene by *S. aureus*, which added to bacteria another new mechanism for resistance to antibiotics, especially methicillin, which is a criterion for diagnosing MRSA isolates molecularly, as it is considered an inevitable criterion for confirming MRSA (Lee *et al*., 2004). As mentioned previously, the results of the study were based on diagnosis and characterization by molecular methods in order to obtain the best and most accurate and sensitive results.

The detection and diagnosis of *S. aureus* and MRSA bacteria depend on traditional and molecular methods (Paterson *et al.*, 2014).

The direct contact of poultry farm workers with birds during field management operations is an important factor in the transmission of *S. aureus* from poultry to farm workers and vice versa (Assafi *et al*., 2020), and therefore, *S. aureus* isolated from poultry is considered a risk indicator for humans living near poultry farms or dealing with them or with their production chains, as confirmed by the researcher (Wertheim *et al*., 2005; Miro and Jwher, 2025). This may also occur due to the failure of biosecurity procedures, which include cleaning and sterilization of field components from chicken remains, or through handling birds for therapeutic purposes (Hussein *et al*., 2015). Therefore, reversing transmission from workers can also cause contamination and transmission of *S. aureus* to birds and their environment if biosecurity procedures are not applied properly (Geenen *et al*., 2012; Hussein *et al*., 2015; Assafi *et al*., 2020; Rahma and Jwher, 2024; Miro and Jwher, 2025).

Isolation and prevalence data vary and differ between different studies at the local and international levels. However, comparisons can still be made, noting that there are specific factors resulting from substantial differences in sampling techniques, sample types, sampling seasons, geographic locations, and samples number. This constituted a significant shortcoming in this study due to the high cost of analysis, the geographical area studied, and the difficulty of obtaining data.

Regarding the most common virulence genes in MRSA-positive samples, *fnbB* was followed by *hlg*, representing 63.2% and 60.5% of the total samples, respectively, while the least common genes were *icaA* and *ebpS*, representing 57.9% and 50%, respectively.

The highest virulence gene registration (100%) was for the *fnbB* gene from chicken and grass isolates, *hlg* gene was highest in isolates from air vents, water, and feed, *icaA* gene was highest in isolates from soil and chicken feed, and *ebpS* gene was highest in isolates from grass and chicken feed. These results differ from those reported by Sonola *et al.* (2023).

The present study also revealed that the virulence genes *fnbB, icaA, hlg*, and *ebpS* were found to be frequent in 37% of bacterial isolates, followed by *fnbB, ica A,* and *ebp*S in 18% of isolates, and the *fnbB* and *ebpS* genes were found to be the least frequent, occurring in 3% of isolates. As for the *icaA* gene, it is responsible for encoding virulence genes that produce biofilms, which have significant impacts on the development of (MRSA) infections (Abdrabaa *et al.*, 2023).

The presence of virulence genes in these isolates indicates their ability to cause infections that may be difficult to treat, in addition to considering all components of the poultry environment that were under study as potential reservoirs for them.

Chapter Six

Conclusions and Recommendations

6-1: Conclusions:

1- There is a major deficiency in applying the biosecurity measures in most poultry farms in northern Iraq.
2- Most cases of failure and decline in biosecurity measures in poultry fields occurred with an increasing age of the chicken.
3- Assessing the prevalence of *S. aureus* in poultry flocks is crucial for assessing future risks in poultry production and associated occupational hazards.
4- The isolation rate of 43.16% is considered an indicator of high contamination with *S. aureus*.
5- The virulence genes *fnbB*, *icaA*, *hlg*, and *ebpS* were common in most isolates and are from different sources.
6- The possession of virulence genes by bacterial isolates indicates their ability to cause infection.

6-2: Recommendations:

1- Educating breeders about the need to implement biosecurity measures, explaining their importance in maintaining poultry health and reducing economic losses.
2- Mapping the biosecurity level to identify areas at risk of disease spread. This procedure is useful in cases of epidemic disease outbreaks and facilitates the implementation of surveillance strategies.
3- Genetic analysis of all *S. aureus* strains from different sources to determine their relationship to the potential transmission of resistant bacteria to humans through the consumption of poultry products containing these strains.
4- Conduct future studies to determine the prevalence of MRSA in a wider area of Iraq.
5- Further studies are being conducted to investigate other types of bacteria, such as *E. coli* and Salmonella, which will help assess the health risks to humans.
6- Study of the contamination level of *Staphylococcus aureus* in relation to Infectious Bursal disease and Newcastle disease in poultry.

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الخلاصة

على الرغم من الاهتمام الكبير الذي تحظى به صناعة الدواجن في العراق، إلا أنها لا تزال تعاني من مشاكل متعددة أدت إلى خسائر اقتصادية فادحة، من بينها المكورات العنقودية الذهبية، التي تسبب أمراضًا عديدة مثل التهاب المفاصل وتسمم الدم، بالإضافة إلى التسمم الغذائي لدى البشر. هدفت هذه الدراسة إلى تقييم مستويات تدابير الأمن الحيوي في مزارع الدواجن من خلال تقييم انتشار التلوث بالمكورات العنقودية الذهبية في حقول الدواجن اللاحم، وتحديد المكورات العنقودية الذهبية المقاومة للميثيسيلين، فضلا عن توفير بيانات وبائية حول انتشار هذه الكائنات الدقيقة في الدجاج وبيئتها المحيطة، بالإضافة الى الكشف عن جينات الفوعة للعزلات الجرثومية.

استهدفت الدراسة وبصورة عشوائية 26 مزرعة دجاج لحم في محافظات دهوك ونينوى وأربيل في شمال العراق. تم اعتماد استمارة استبيان للتحقق من تطبيق إجراءات الأمن الحيوي في مزارع الدواجن ضمن مناطق الدراسة المختلفة، لثلاثة محاور رئيسية شملت: التنظيف والتطهير، والعزل، والمراقبة، وذلك خلال الفترة من سبتمبر2024 إلى يناير 2025 ، كما تم جمع 234 مسحة وعينة، شملت أيدي العمال وجلد الدجاج ومفرغات الهواء والمعالف ومصادر المياه والتربة والفرشة والعشب وأعلاف الدجاج، من كل مزرعة، ثم نقلت مباشرة إلى مختبر البحوث العلمية في كلية الطب البيطري / جامعة الموصل لإجراء الفحوصات المخبرية البكتريولوجية اللازمة.

استخدمت الطرق التقليدية لعزل وتحديد جراثيم المكورات العنقودية الذهبية، بما في ذلك الزراعة على الأوساط الانتخابية والاختبارات الكيموحيوية. استخدم وسط الاكار الكروموجيني لتشخيص المكورات العنقودية المقاومة للميثيسلين، بينما استخدمت الطرق الجزيئية للكشف عن جين ***nuc*** لتحديد جراثيم المكورات العنقودية الذهبية وجين ***mec A*** لـتحديد المكورات العنقودية المقاومة للميثيسلين. استخدم اختبار ارتباط بيرسون لإيجاد العلاقة بين تطبيق إجراءات الأمن الحيوي ونسبة العزل لجراثيم المكورات العنقودية الذهبية. تم الكشف أيضًا عن جينات وعوامل الضراوة ***hlg*** و ***ebpS*** و ***fnbB*** و ***ica*** بين عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA)

أظهرت نتائج عزل المكورات العنقودية الذهبية أن 101 عينة من أصل 234 عينة كانت إيجابية، أي بنسبة 43.16% من إجمالي العينات المشمولة في الدراسة (أيدي العمال، الدجاج، مفرغات الهواء، المعالف والماء، التربة، الفرشة، العشب، وأعلاف الدجاج). سُجِّلت أعلى نسبة عزل من أيدي العمال والدجاج بنسبة 53.85%، وأقل نسبة عزل من المعالف والماء بنسبة

34.61%، وسُجِّلت أعلى نسبة انتشار للمكورات العنقودية الذهبية بين عينات من حقول ومناطق مختلفة من أيدي العمال والدجاج بنسبة 13,86%، بينما سُجِّلت أقل نسبة انتشار من المعالف والماء بنسبة 8,91%. سُجِّل أعلى معدل انتشار في محافظة دهوك، تلتها أربيل ثم نينوى، بنسبة 50.50%، و37.62%، و11.88%، على التوالي.

وفيما يتعلق بإجراءات الأمن الحيوي، سُجِّل تطبيق 19 فئة من إجراءات الامن الحيوي، أي ما نسبته 86.36%، في الحقل رقم 6 بعمر 7 أيام، بالمقابل كانت نسبة العزل 0%، على العكس من ذلك، في الحقل رقم 22 بعمر 40 يومًا وثَّق تطبيق 5 فئات من إجراءات الامن الحيوي، بنسبة 22.73%، وسجل نسبة عزل 100% في العينات المدروسة.

أظهرت نتائج عزل المكورات العنقودية الذهبية المقاومة للميثيسيلين أن 38 عينة من أصل 101 عينة من المكورات العنقودية الذهبية كانت إيجابية، أي بنسبة 37.62%. سُجلت أعلى نسبة انتشار للمكورات العنقودية الذهبية المقاومة للميثيسيلين بين عينات من حقول ومناطق مختلفة من فرشة الدجاج والدجاج، بنسبة 23.68% و18.42%، على التوالي، بينما كانت أقل نسبة انتشار في عينات العشب والتربة وأعلاف الدجاج، بنسبة 2.63% و5.26% و5.26% على التوالي. كشفت الدراسة عن وجود جينات *fnbB* و *ica* A و *hlg* و *ebp S* بنسبة 63.2% و57.9% و60.5% و50%، على التوالي، من أصل 38 عينة من المكورات العنقودية الذهبية المقاومة للميثيسيلين ومن المصادر المختلفة.

تشير معدلات العزل العالية للمكورات العنقودية الذهبية والمكورات العنقودية الذهبية المقاومة للمثيسيلين، إلى جانب عوامل الضراوة الخاصة بها، إلى قدرتها على التسبب في عدوى قد يكون من الصعب علاجها، على اعتبار ان جميع مكونات بيئة الدواجن قيد الدراسة خزانات محتملة للجراثيم.

دراسة وبائية للمكورات العنقودية الذهبية مع جينات الضراوة ذات الصلة من حقول الدجاج

رسالة ماجستير

تقدم بها

رمضان فتاح ميرو عبد الرحمن

الى

مجلس كلية الطب البيطري

جامعة الموصل

كجزء من متطلبات درجة الماجستير

في اختصاص الطب البيطري/ الصحة العامة البيطرية

باشراف

الأستاذ الدكتور ضياء محمد طاهر جوهر

2025 م **1447هـ**

جامعة الموصل

كلية الطب البيطري

دراسة وبائية للمكورات العنقودية الذهبية مع جينات الضراوة ذات الصلة من حقول الدجاج

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في اختصاص الطب البيطري/ الصحة العامة البيطرية

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