**Faculty of Graduate Studies**

**An-Najah National University**

# Prevalence and Characterization of *Staphylococcus aureus* Isolated from Bulk Tank Milk Dairy Cow Farms in West Bank-Palestine

**By**

**Haya Isayed**

**Supervisor**

**Dr. Ghaleb Adwan**

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**This thesis was defended successfully on 18/3/2018 and approved by:**

| **Defense Committee Members** | **Signature** |
|---|---|
| **1. Dr. Ghaleb Adwan (Supervisor)** | **……** |
| **2. Dr. khaled Qabaha (External Examiner)** | **……** |
| **3. Dr. Motasem Al-Masr (Internal Examiner)** | **……** |

# Dedication

I dedicate thesis to my husband Yousef and my children Majeed and Tareza, my Mother, Father, Sisters, Brother, all the family members and friends, my anti Nasra and anti Samya.

To my Grandmother’s Soul and the priest Ibrahim soul.

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I would thank my husband, my family, specially my mother and father for their continuous love and support in my decisions and my life.

Thank you all …

# الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

**Prevalence and Characterization of *Staphylococcus aureus* Isolated from Bulk Tank Milk Dairy Cow Farms in West Bank-Palestine**

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

# Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

**Student's Name:** **اسم الطالب:**

**Signature:** **التوقيع:**

**Date:** **التاريخ:**

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# List of Abbreviations

| | |
|---|---|
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MSSA | Methicillin-sensitive *Staphylococcus aureus* |
| ERIC-PCR | Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction |
| spA | Staphylococcal protein A (*spa)* |
| S.aureus | *Staphylococcus aureus* |
| BTM | Bulk Tank Milk |
| MDR | Multi-drug resistant |
| PBP | Penicillin-binding proteins |
| SCC*mec* | Staphylococcal cassette chromosome |
| HA-MRSA | Health care-associated |
| CA-MRSA | Community-associated |
| LA-MRSA | Livestock-associated |
| Ig-binding protein A | Immunoglobulin-binding proteinA |
| MLST | Multilocus sequence typing |
| MLEE | Multilocus enzyme electrophoresis |
| REP | Repetitive extragenic palindromic |
| PVL-gene | Panton–Valentine leukocidin gene |
| PFGE | Pulsed Field Gel Electrophoresis |
| TSB | Tryptone Soy Broth |
| MSA | Mannitol salt agar |
| NA | Nutrient agar |
| MHA | Meullar-Hinton agar |
| DA | Clindamycin |
| CN | Gentamicin |
| VA | Vancomycin |
| E | Erythromycin |
| FOX | Cefoxitin |
| SXT | Trimethoprim/Sulfamethoxazole |
| CIP | Ciprofloxacin |
| IMP | Imipenem |
| NA | Nalidixic acid |
| MEM | Meropenem |
| AK | Amikacin |
| TE | Tetracycline |
| CLSI | Clinical and Laboratory Standard Institute |
| UPGMA | Unweighted pair group method for arithmetic averages |
| S | Susceptible |

| | |
|---|---|
| I | Intermediate |
| R | Resistant |
| L | Ladder |
| NC | Negative control |

**Prevalence and Characterization of *Staphylococcus aureus* Isolated from Bulk Tank Milk Dairy Cow Farms in West Bank-Palestine**

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## Abstract

The emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in cattle is considered one of the major public health problem. The current study aimed to characterize and document the prevalence and distribution of MSSA (Methicillin-sensitive Staphylococcus) and MRSA in bulk tank milk samples (BTM), antibiotic resistance rate, and genetic characterization of clones for both MSSA and MRSA using ERIC-PCR and *spA* typing. In addition, to evaluate the occurrence of MRSA in human workers in these cow dairy farms and in the environment of these farms, this will provide useful data for epidemiological studies. A total of 57 bovine BTM samples, 45 dust samples from the environmental surfaces and 16 nasal swabs from the human workers in these farms were collected from 12 bovine diary farms in Jenin district in northern West Bank-Palestine, between September and October, 2017. A total of 251 of *S. aureus* isolates were recovered from all samples, which were 136 (54.2%) isolates detected from bulk tank milk samples, 35 (14%) isolates from nasal human workers and 80 (31.8%) isolates recovered from environment sources. Only 68 of *S. aureus* isolates were tested for antibiotic resistance, 39 isolates were from

BTM samples, 10 isolates were from human workers nasal swabs and 19 isolates from environment sources. Results showed that 58.3% (7/12) of farms had at least one BTM sample contaminated with MRSA. In addition, *S. aureus* in this study was detected in 68.4% bovine BTM samples, while MRSA was detected in 45.6% of bovine BTM samples. *Staphylococcus aureus* recovered from BTM samples, environment sources and nasal human workers had a high level of resistance against many different antibiotics. Results in current study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were multi-drug resistant (MDR). ERIC-PCR profile and *spa* typing showed that some strains of the same clone had been isolated from different sample sources in different farms. For example, *spa* type t164 was isolated BTM samples from different farms and human worker nasal swab. This evidence suggested that these strains of the same clone or *spa* type could be circulated between cattle and humans.

Results of this study showed that Palestine BTM samples is a common source of MRSA. Special attention to basic hygiene, good husbandry and biosecurity measures on bovine dairy farms have a tendency to reduce the spread of MRSA in animal population. Careful monitoring of the resistance status of *S. aureus* in animals, humans and in dairy farm environments is required due to some clones that circulate between them.

# Chapter One
# Introduction

## 1.1. General Background

### 1.1.1. *Staphylococcus aureus*

Taxonomically, the genus *Staphylococcus* is in the bacterial family *Staphylococcaceae*. Staphylococci are Gram-positive spherical bacteria, which usually arranged in a grape-like clusters, facultative anaerobes, catalase-positive, oxidase-negative and usually unencapsulated. This genus of bacteria has more than 30 species. *Staphylococcus aureus* (*S. aureus*) is considered the most clinically significant species in this genus. It has large, round, golden-yellow colonies, often with β-hemolysis, when grown on blood agar (Brooks *et al*., 2001).

*Staphylococcus aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. It was estimated that approximately 20-30% of the human population are *S. aureus* carriers. This organism expresses many potential virulence factors including: surface proteins that promote colonization of host tissues, invasins that promote bacterial spread in tissues, surface factors that inhibit phagocytic engulfment, biochemical properties that enhance their survival in phagocytes, immunological disguises, membrane-damaging toxins that lyse eukaryotic cell membranes, exotoxins that damage host tissues or otherwise provoke symptoms of disease and inherent and acquired resistance (Dinges *et al*., 2000; Brooks *et al*., 2001).

In humans, *S. aureus* can cause a diverse range of diseases ranging from relatively minor skin infections to serious and life-threatening infections such as endocarditis, pneumonia, and sepsis (Paterson *et al*., 2014a). In addition, in dairy animals including cows this pathogen is considered one of the most common causative agent of clinical and sub-clinical mammary infections (Haran *et al.*, 2012).

Emergence of antibiotic-resistant zoonotic bacterial pathogens in farm animals represents a major public health problem for both humans and animals. This is due to the use of antimicrobial agents on dairy farms as well as in other food animal production systems. In this case, antimicrobial agents are used to treat highly prevalent infections among cows, such as subclinical mastitis, and as a preventive measure during dry cow therapy. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential (Piddock, 1996). Resistance mechanisms in *S. aureus* include alteration of the target with decreased affinity for the antibiotic, trapping of the antibiotic and efflux pumps and rarely of the enzymatic inactivation of the antibiotic (Pantosti *et al.*, 2007). Different antibiotic classes of drugs are used in animal health management and in human medicine, the selection of resistance to one drug class may lead to cross-resistance to another. Beta-Lactams bind to the penicillin-binding proteins (PBP) essential for cell wall biosynthesis and inhibit peptidoglycan crosslink formation, leading to bacterial cell lysis. Resistance to β-lactams in Methicillin-resistant *S. aureus* (MRSA) is conferred by the acquisition of a mobile genetic element, the

staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene, which encodes PBP2α, which has reduced affinity for β-lactam antibiotics. As a result, cell wall biosynthesis in MRSA strains continues even in the presence of otherwise inhibitory levels of β-lactam antibiotics (Paterson *et al*., 2014a; Gopal and Divya, 2017). Recently, a divergent homolog, $mecA_{LGA251}$ has been described in Garcia-Alvarez *et al*. (2011) and in Paterson *et al*. (2012) that approximately 70% similarity at the DNA level to the classical *mecA* gene, and now is officially identified as *mecC* (Ito *et al*., 2012). Methicillin-resistant *S. aureus,* which is almost resistant to all types of β-lactam antibiotics has emerged as a major cause of health care-associated (HA-MRSA) and community-associated (CA-MRSA) infections. In addition, other clonal complexes of livestock-associated (LA) MRSA have been emerged among different animals such as pigs, cattle, poultry, farmers and veterinarians in different countries (Haran *et al*., 2012; Köck *et al*., 2013; Paterson *et al*., 2014a; Luini *et al*., 2015), and these clonal complexes (mainly Clonal Complex 398 (CC 398) ) of LA-MRSA clone have ability to cause infections in both humans and animals. The presence of MRSA in both bovine milk and dairy environments plays a major potential risk to farm workers, veterinarians and farm animals that are exposed to contaminated cattle (Lee, 2003; Juhász-Kaszanyitzky *et al*., 2007).

### 1.1.2. Staphylococcal protein A (spa)

Staphylococcal protein A (spA) or surface Ig-binding protein A is considered one of the most important virulence factor encodes by *S. aureus*. The function of this protein is to capture IgG molecules in the inverted orientation and therefore avoid phagocytosis process of the bacterial cells by the host immune response. Typing the highly variable Xr region of the *spA*-gene is one of the most common methods used to classify *S. aureus* strains and known as *spa* types. Even if well-established genotyping methods like multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MLEE) are indispensable (Koreen *et al*., 2004), *spa*-typing method has advantages due to its high discriminatory power, typing accuracy, speed, reproducibility, ease of interpretation, and also facilitates communication and data comparison between national and international clinical laboratories (Votintseva *et al*., 2014)

The *spA* extracellular domains are subject to immune surveillance, which is expected to increase its speed of evolution (Stephens *et al*., 2008). Consequently, there are currently 17625 *spa* types in the Ridom SpaServer database (http://spaserver.ridom.de).

### 1.1.3. Enterobacterial Repetitive Intergenic Consensus (ERIC) - PCR

In ERIC-PCR, the primers were designed to known repetitive and highly conserved target DNA sequence in some bacterial genome DNA. Thus, it is considered a PCR-fingerprinting technique but not arbitrary PCR. In bacterial genome, it has been reported that there are few repetitive

sequences including ERIC sequences, repetitive extragenic palindromic (REP) sequences, and BOX elements. The ERIC sequences are available in many copies in the genomes of different Gram-negative such as *Escherichia coli*, *Salmonella typhimurium* (Hulton *et al*., 1991; Zulkifli *et al*., 2009). These elements are highly conserved at the nucleotide level, 126 bp long, and include a central core inverted repeat. The position of ERIC sequences in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic *et al*., 1991; Son *et al.*, 2002). In ERIC-PCR a band pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences. This technique uses consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements such as ERIC sequence for subtyping Gram-negative enteric bacteria (Hulton *et al.*, 1991; Zulkifli *et al*., 2009).

## 1.2. Literature Review

Methicillin-resistant *S. aureus* has increasingly been recognized in farm animal populations in recent years and has been considered as an important pathogen in animals.

The overall prevalence of MRSA in bovine milk from both healthy and infected cows has been reported from several countries. In USA and Denmark, the prevalence of *S. aureus* isolated from bulk tank milk (BTM) samples in USA and Denmark was (60%) and (55%), respectively.

Significant differences between the two countries were detected in nine antimicrobials. Denmark had a higher probability of having reduced susceptibility to ciprofloxacin and streptomycin. The United States isolates had a higher probability of having reduced susceptibility to seven other antimicrobial agents bacitracin, gentamicin, kanamycin, penicillin, sulphamethoxazole, tetracycline, and trimethoprim (Sato *et al*., 2004). In another study from USA, the herd prevalence of *S. aureus*, including MRSA, was estimated from BTM samples from Minnesota farms. A total of 150 pooled BTM samples from 50 farms, were assessed. Herd prevalence of Methicillin-sensitive *S. aureus* (MSSA) was 84%, while MRSA herd prevalence was 4%. Bulk tank milk sample prevalence of MSSA and MRSA was 62% and 1.3%, respectively (Haran *et al*., 2012). Antibiotic susceptibility testing of MSSA isolates showed that 58% were susceptible to all tested antibiotics and 5.4% were resistance to 3 or more antibiotics classes, while the 2 MRSA isolates displayed resistance to β-lactams, cephalosporins, and lincosamides and were multi-drug resistant (MDR). Staphylococcal protein A gene (*spa*) typing identified *spa* types t529 and t034 most frequently among MSSA isolates, while t121 type was detected in MRSA isolates. Seven isolates, including the 2 MRSA isolates were staphylococcal enterotoxins B, C, D, and E producers (Haran *et al*., 2012).

In Great Britain, the prevalence rate of *mecC* MRSA isolated from bovine BTM samples in England and Walse was 2.15% but not in Scotland. A total of 70% of *mecC* MRSA isolates were belonged to a sequence type

(ST) 425, while the other 30% *mecC* MRSA isolates belonged to clonal complex CC130. Resistance to non-β-lactam antibiotics was uncommon (Paterson, 2014b). In Norway, *S. aureus* was detected in 75% bovine BTM samples, production of enterotoxins was observed in approximately 22% of these isolates (Jørgensen *et al*., 2005). In Switzerland, the prevalence was 1.4% and the strains showed that they belonged to *spa* type t011, SCC*mec* type V and were resistant to ampicillin, cefoxitin, clindamycin, erythromycin, oxacillin, penicillin and tetracycline (Huber *et al.,* 2010). In Belgium, about 10% of the farms suffering from *S. aureus* mastitis have an MRSA problem and the in-herd prevalence varied between 0% and 7.4%. These MRSA strains showed that they were all resistant to tetracycline and frequently resistant to macrolides, lincosamides and aminoglycosides. These strains were ST398, *spa*-types t011 or t567 and had *SCCmec*-IVa or V, indicating that these belong to the emerging livestock-associated MRSA (LA-MRSA) strains of CC398 (Vanderhaeghen *et al*., 2010). In southwest Germany, (5.1 to 16.7%) of dairy cows were found positive for MRSA and in all tested herds MRSA of *spa*-type t011 were detected in milk samples (Spohr *et al*., 2011). In Italy, the prevalence of MRSA among *S. aureus* isolates was 9.2%. The prevalence rate of MRSA strains was 14.75% among tested herds. The most frequently identified clonal complex among tested isolates was CC8, which included 44.6% of MSSA strains from 22 out of 61 herds, while 66.7% of strains belonged to CC398 and the remaining strains were assigned to other clonal complexes. The characterization of the SCC*mec* cassettes revealed 3 different types IV, V

and IV/V. Results of *spa* typing showed an overall similar profile of the strains belonging to the same CC: t127-CC1, t1730-CC97, t899 in 8 out of 10 CC398. In the remaining 2 isolates a new *spa* type, t14644, was identified. Enterotoxin and leukocidin genes were carried only by CC1, CC8 and CC97-MRSA. Approximately, 86% of MRSA strains were phenotypically resistant to all β-lactams. All MRSA strains showed an intermediate or complete resistance to thiamphenicol, while the other antibiotics displayed different patterns of sensitivity, independently of the clonal complex. All but the CC8 and CC1 strains, also carried the tetracycline resistance gene and the CC398-MRSA-IV strains harboured in addition the florfenicol exporter gene. The gene conferring resistance to streptogramin was observed in 73% of MRSA strains (Luini *et al*., 2015). In other study from the same country, the dairy herd prevalence rate for *S. aureus* and MRSA using bovine BTM samples was 47·2% and 3·8%, respectively. MLST showed that the majority 87.5% of isolates belonged to the typical livestock-associated lineages: ST398, ST97 and ST1. Other sequence types were reported such as ST3211. All strains carried SCC*mec* cassette types IV or V. Most of the MRSA strains were resistant to tetracycline 93.7% and MDR 90.6%, 40.6% of MRSA isolates were microbiologically and clinically resistant to fluoroquinolones (Cortimiglia *et al.,* 2016).

In Japan, according to Hata *et al*. study in 2010, the prevalence of MRSA was 1.5% these isolates showed geno- and serotypes that were identical or similar to those of human MRSA isolates in this country (ST5,

staphylococcal cassette chromosome *mec* type II [SCC*mec* II], *spa* type t002 or t375, and coagulase serotype II, and ST89, SCC*mec* IIIa, *spa* type t5266, and coagulase serotype I) (Hata *et al*., 2010). In Iran, the prevalence of *S. aureus* was 16% in raw cow tank bulk milk samples, 75% of these isolates were enterotoxigenic (Rahimi and Alian, 2013). Other study from the previous country, showed that the prevalence of MRSA among raw milk samples collected from bovine was 20%. Totally, *SCCmec IV* and *SCCmec V* types were the most prevalent alleles in these MRSA isolates. Detection of *SCCmec* types IV and V suggested the emergence of CA-MRSA strains in this geographical area and occurrence of *SCCmec* I and II alleles indicated a possible transmission of MRSA from humans to animals (Khaji and Shahreza, 2017). In India, the prevalence of MRSA among *S. aureus* isolates was 13%. These isolates also were highly resistant to different antibiotics and a significant variation in the expression of virulance factors was observed in these isolates (Kumar *et al*., 2011). In korea , 402 *S. aureus* isolates were recovered from bovine mastitis milk during 2003-2009. All isolates were susceptible to pirlimycin, telithromycin, novobiocin, penicillin/novobiocin, quinupristin/dalfopristin, clindamycin, rifampin, ciprofloxacin, trimethprim/sulfamethoxazol, vancomycin, and linezolid. But 66% of the *S. aureus* isolates were resistant to penicillin, 11.9% to gentamicin, 7.7% to erythromycin and 4.2% to tetracycline. The prevalence rate of MRSA among *S. aureus* isolates was 6.2% (Nam *et al.*, 2011). In Turkey, the prevalence of MRSA among MSSA strains isolated from bovine milk with mastitis was 17.2%.

All MRSA strains were MDR. The resistance rates to the antimicrobials tested were 100%, 100%, 100%, 100%, 93.75%, 83.75% and 43.75% for erythromycin, clindamycin, chloramphenicol, gentamicin, tetracyclin, ciprofloxacin and vancomycin, respectively. The pulsed-field gel electrophoresis characterization showed the presence of three pulsotypes with their variants. The pulsotype B strains were type IV with *SCCmec* typing, *spa* type t190 and MLST type ST8. The strains with pulsotype A and C were *SCCmec* III, *spa* type t030. The MLST type of pulsotype A was ST239 and pulsotype C was one allele variant of ST239. None of the MRSA isolates had the PVL gene. Presence of hospital-related MRSA strains may indicate transmission of these strains between human and animals (Turkyilmaz *et al*., 2010).

In Brazil, The prevalence rate of *S. aureus* in milk produced in 37 farms were analyzed. *Staphylococcus aureus* strains were detected in 18 (7.3%) milk samples: 14 (6.7%) from samples of individual cows, and 4 (10.8%) from BTM. Prevalence of enterotoxigenic *S. aureus* was 14.3% and 50% in *S. aureus* isolated from individual milk samples and BTM samples, respectively. PFGE (Pulsed Field Gel Electrophoresis) analysis revealed a great genetic heterogeneity among *S. aureus* strains isolated from raw milk (Fagundes *et al*., 2010). In other study from the same previous country, the prevalence rate of *S. aureus* isolated from milk samples collected from mammary glands positive for subclinical mastitis was 52.1%. The presence of *mecA* gene was detected in 48.3% of *S. aureus* isolates. Of the *S. aureus*

isolates, 23.3% were MRSA and 25.0% were oxacillin-susceptible *mecA*-positive *S. aureus* (OS-MRSA) (Guimarães *et al*., 2017).

In Ethiopia, *S. aureus* was isolated from 38%, 33%, and 66% of the milk samples originating from the dairy farms, the milk retailers, and the milk collection centers, respectively, 26% of the isolates were resistant to oxacillin (Tolosa *et al*., 2016).

### 1.3. Aims of the Study

The prevalence and characterization of MSSA and MRSA isolated from bovine BTM samples have not been examined previously in west bank-Palestine. The current study aimed to characterize and document the prevalence and distribution of MSSA and MRSA in BTM samples, antibiotic resistance rate, and genetic characterization of clones for both MSSA and MRSA. In addition, to evaluate the occurrence of MRSA in dairy environment surfaces and human workers in contact with bovine dairy farms, this will provide useful data for epidemiological studies.

# Chapter Two
# Materials and Methods

## 2.1. Sample Collection and *S. aureus* Identification

A total of 57 bovine BTM samples in this study were collected from 12 bovine dairy farms in Jenin district (Zababdeh, Qabatiya, Mirka, Selat Al-daher, Arrabah, Zayet Abu-Elwafa and Ajja) in northern West Bank-Palestine, between September and October, 2017. The total number of cows in these farms was 1343. All dairy cattle in these farms are milked by machines. Other 16 nasal swabs were collected from the human workers in these farms during the same period of BTM sample collection. Additionally, 45 dust samples from the environmental surfaces were collected by sterile swabs. Environmental surfaces include machines, cow dung or manure, feeders, ventilation ducts, milking pipeline and farm walls. The samples immediately were kept refrigerated in a container containing ice cubes and transferred to the Microbiology laboratory at An-Najah National University-Nablus, Palesine, for culturing and identification. Data about number of cows in each farm and number of samples obtained from each farm are presented in Table 3.1. All samples were cultured into 7 mL TSB and incubated for 18-24h at 37ºC. Then, 10 µl of TSB (Tryptone soy broth) was subcultured by streaking method on Mannitol salt agar (MSA). Then 3-5 separated yellow colonies were subculture on nutrient agar (NA) for further analysis to identify *S. aureus* depending on morphological and biochemical characteristics such as Gram stain, catalase test, and hemolytic reaction and coagulase test.

## 2.2. Media Preparation

### 2.2.1. Nutrient agar (NA)

Nutrient agar (ACUMEDIA, USA) was prepared according to manufacturer's instructions labeled on the bottle. In a 0.5 L bottle, a 5.75 g NA and 245 ml deionized water were heated and mixed until NA was dissolved. The solution was allowed to boil for 1min, and then autoclaved at 121°C for 15min. After that it was allowed to cool to about 55°C. The agar was poured into sterile Petri dishes to have about 20 ml each and left overnight at room temperature. In next day, the Petri dishes were kept at 4-6°C in refrigerator.

### 2.2.2. Nutrient broth (NB)

Nutrient broth (ACUMEDIA, USA) was prepared according to manufacturer's instructions labeled on the bottle. In a 0.5 L bottle, 2 g of NB and 248 ml deionized water were mixed and dissolved well. The broth was then distributed into tubes to have 7 ml each and plugged with cotton. The tubes were autoclaved at 121°C for 15min, allowed to cool and then kept at 4-6°C in refrigerator.

### 2.2.3. Mannitol salt agar (MSA)

Mannitol agar (BD, USA) was prepared according to the manufacturer's instructions labeled on the bottle. A 1 L bottle containing 27.75 g of Manitol salt agar and 473 ml deionized water were heated and mixed well until the agar was dissolved. The mixture was allowed to boil for 1 minute,

and then autoclaved at 121°C for 15min. After that it was allowed to cool to approximately 55°C, and the agar was poured into sterile Petri dishes to have approximately 20 ml each and left overnight at room temperature. In the next day, the Petri dishes were kept at 4-6°C in refrigerator.

### 2.2.4. Meullar-Hinton agar (MHA)

Meullar-Hinton agar (HIMEDIA, INDIA) was prepared according to the manufacturer's instructions labeled on the bottle. A 2L bottle containing 38 g of MHA and 962 ml of deionized water missed well and heated until the agar was dissolved. The mixture was allowed to boil for 1 minute, and then autoclaved at 121°C for 15min. After that it was allowed to cool to 55°C, the agar poured into sterile Petri dishes to have approximately 25-30 ml each and left overnight at room temperature. In the next day, the Petri dishes were kept at 4-6°C in refrigerator.

### 2.2.5. Blood agar

Blood agar was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 20 g blood agar base (Oxoid, USA) and 455 ml deionized water were heated and stirred until the agar was dissolved. Then, the mixture was autoclaved at 121°C for 15 min and was allowed to cool to about 50°C-50°C. After that, 25 ml of sterile defibrinated sheep blood was added aseptically and mixed thoroughly. Then, the agar was poured into Petri dishes to have 20-25 ml each, then covered and left overnight at room temperature. In the next day, the Petri dishes were store at 4-6°C in refrigerator.

### 2.2.6. Tryptone soy broth (TSB)

Tryptone soy broth (HIMEDIA, INDIA) was prepared according to manufacturer's instructions labeled on the bottle. In a 1 L bottle, 15 g TSB and 485 ml deionized water were mixed well and boiled to dissolve the agar. The mixture was then distributed into tubes to have 7 ml each and plugged with cotton. The tubes were autoclaved at 121°C for 15 minutes, allowed to cool and then kept at 4-6°C in refrigerator.

## 2.3. *Staphylococcus aureus* Identification

*Staphylococcus aureus* isolates recovered in this study were identified and confirmed by the following tests:

### 2.3.1. Gram staining

Gram staining was performed to distinguish Gram-positive bacteria from Gram-negative bacteria. A thin smear of bacteria was made on a clean glass slide by picking the isolates from marked colonies after 24 hours incubation on Meuller-Hinton agar plate and mixed with a drop of distilled water. The smear was heat fixed by passing through a flame 2 or 3 times. Care was taken to avoid air bubbles formation and overheating to prevent distortions of the glass slide. After cooling, the slide was flooded with primary stain (crystal violet) and left for one minute then washed with tap water. Gram's iodine solution was then added for one minute and again washed off with tap water, decolorized with acetone alcohol from 10 to 20 seconds and then again washed with tap water. Finally, the slide was

flooded with a counter stain (safranin) for 1 minute then washed with tap water and let to dry. The smear was observed under 100x objective lens of the light microscope (Cappuccino and Sherman, 1996)

### 2.3.2. Catalase test

Catalase test was carried out by addition 1-2 drops of 3% hydrogen peroxide on bacterial colony cultured on nutrient agar (Cappuccino and Sherman, 1996).

### 2.3.3. Subculture on mannitol salt agar

Aseptically, 10 µl of TSB was subcultured by streaking method on Mannitol salt agar (MSA), the plate was incubated for 24 hours at 37ºC. Then 3-5 separated yellow colonies were subculture on nutrient agar (NA) for further analysis.

### 2.3.4. Slide coagulase test

A dense suspension of Staphylococci from NA and one drop of citrated plasma were prepared on slide, the suspension was mixed well. Agglutination or clumping of cocci within 5-10 seconds is taken as positive (Cappuccino and Sherman, 1996). Negative samples were tested by tube coagulase test.

### 2.3.5. Tube coagulase test

This test was carried out by inoculating 1ml of diluted (1:4) fresh citrated human plasma with a colony from pure culture of Gram positive cocci in

grape-like clusters that is both catalase- and mannitol fermentation positive. Then, the tube was incubated at 37ºC for 3 h and was checked during that time, if the result is negative the tube was incubated for18-24 hours at 37ºC (Cappuccino and Sherman, 1996).

### 2.3.6. Hemolytic reaction

All the isolates were tested for hemolysis after overnight incubation at 37°C on sheep blood agar. Hemolysis was recorded as α-hemolysis (greenish discoloration that surrounds a bacterial colony**)**, β-hemolysis (clear zone around colony), and negative γ-hemolysis. All *S. aureus* strains tested produce β-hemolytic reaction.

### 2.4. Antibiotic Susceptibility Test

Antimicrobial susceptibility for 68 *S. aureus* strains were determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2016). Antibiotic resistance was carried out using the following antibiotic disks (Oxoid): clindamycin (DA, 2 µg); gentamicin (CN, 10µg); vancomycin (VA, 30 µg); erythromycin (E, 15 µg); cefoxitin (FOX, 30µg); Trimethoprim/Sulfamethoxazole (SXT, 1.25 /23.75µg); ciprofloxacin (CIP, 5µg); imipenem (IMP, 10 µg); nalidixic acid (NA 30µg); meropenem (MEM, 10 µg); amikacin (AK, 30µg) and tetracycline (TE, 30µg). Three to four of 24-hour-old colonies of each *S. aureus* strain were sub-cultured into nutrient broth media and incubated for 3-4 hours with frequent shaking. Mueller Hinton agar (MHA) plates were swabbed with a 4-5-hours-old culture of the bacterial strains, then antibiotic

disks were placed on the MHA plates containing the inoculum. Zones of inhibition was determined after incubation the plates at 35ºC for 18-24 h in accordance with procedures of the Clinical and Laboratory Standard Institute (CLSI, 2016).

Antibitic cefoxitin (FOX 30 μg) was used to detect methicilline resistant *S. aureus*. Zones of inhibition was determined in accordance with procedures of the Clinical and Laboratory Standards Institute (CLSI, 2016), isolates were categorized as susceptible and resistant. According to cefoxitin, *S. aureus* isolates were considered resistant and sensitive if inhibition zones were ≤ 21 mm and ≥22 mm, respectively, after incubation on Mueller Hinton agar at 35ºC for 18-24 h. Methicillin-resistant control strains from our department collection and methicillin-susceptible reference (*S. aureus* ATCC 25923) were used in this study.

## 2.5. DNA Extraction and PCR

### 2.5.1. DNA extraction

*Staphylococcus aureus* genome was prepared for PCR according to method described previously (Adwan et al., 2013). Briefly, cells were scraped off an overnight nutrient agar plate with a sterile loop, re-suspended with 0.8 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), centrifuged for 5 minutes at 11,500 X g rpm, then the supernatant was discarded. Then, the pellet was re-suspended in 0.4 ml of sterile distilled $H_2O$, boiled for 10-15 min, immediately, then, the suspension was incubated on ice for 5-10 min. The debris pelleted by centrifugation at

11,500 X *g* for 5 min. DNA concentration was determined using nanodrop spectrophotometer (Genova Nano, Jenway) and the samples stored at -20ºC until use for further DNA analysis.

### 2.5.2. PCR assay for *mecA* gene detection

The *mecA* gene in 20 MRSA strains selected randomly was detected by PCR with specific primers: *mec*A1 (5'-AAA ATC GAT GGT AAA GGT TGG C-3') and *mec*A2 (5'-AGT TCT GCA GTA CCG GAT TTG C-3'). These primers were used for amplification of a fragment of 532 bp (Adwan *et al*., 2014). Each PCR reaction mix (25 µL) was performed using 12.5 µL of PCR premix with $MgCl_2$ (ReadyMix$^{TM}$ 1.5 U Taq PCR Reaction Mix with 0.2 mM dNTP and 1.5 mM $MgCl_2$, Sigma), 0.3 µM of each primer, and 3 µL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation step at 94ºC for 1 min, followed by 40 cycles of initial denaturation 94ºC for 20 s, 52 ºC for 45 s and 72ºC for 30 s ending with a final extension step at 72 ºC for 2 min. After amplification, the amplified PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and stained with ethidium bromide (0.5 µg/ml).

### 2.5.3. *Spa* amplification

The primer sequences used in this study for amplification of *Staphylococcus* protein A (*spa*) repeated regions were described previously (Prosperi *et al*., 2013). These primers are spa-1113f (5'- TAA AGA CGA

TCC TTC GGT GAG C-3') and spa-1514r (5'- CAG CAG TAG TGC CGT TTG CTT-3'). Each PCR reaction mix (80 µL) was performed using 40 µL of PCR premix with $MgCl_2$ (ReadyMix$^{TM}$ 1.5 U Taq PCR Reaction Mix with 0.2 mM dNTP and 1.5 mM $MgCl_2$, Sigma), 0.3 µM of each primer, and 6 µL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation (4 min at 94°C), followed by 35 cycles of denaturation 94°C for 40 s, annealing 55°C for 40 s, and extension 72°C for 90 s, with a final extension 72°C for 5 min. A total of 15 µl of amplified PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and stained with ethidium bromide (0.5 µg/ml). The remaining amount were purified using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Germaney) according to the user manual, and sequenced by dideoxy chain termination method using ABI PRISM sequencer, model 3130 (Hitachi Ltd, Tokyo, Japan), Bethlehem University, Bethlehem, Palestine. Sequence information was further submitted for accession number in primary bioinformatics web servers.

### 2.5.4. ERIC-PCR typing

The ERIC-PCR was performed using Primer ERIC1: 5`-ATG TAA GCT CCT GGG GAT TCA C-3` and Primer ERIC2: 5-AAG TAA GTG ACT GGG GTG AGC G-3`. Each PCR reaction mix (25 µl) was composed of 10 mM PCR buffer pH 8.3; 3 mM MgCl2; 0.4 mM of each dNTP; 0.8 µM of

each primer; 1.5U of Taq DNA polymerase and 3 µl of DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 2 min at 94ºC, followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 50ºC for 40 s and extension at 72ºC for 1 min, with a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis on 1.7% agarose gel. The gel images were scored using binary scoring system that recorded the absence and presence of bands as 0 and 1, respectively. A binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistical software version 20 (IBM). The number of different bands in each fingerprint was considered for comparison of bacterial species as previously described (Adwan *et al.*, 2016), based on the following criteria: identical clones (no different band), "closely related clones" (have 1 different band), "possibility different clones" (have two different bands), "different clones" (have three or more different bands).

## 2.6. Data Analysis

### 2.6.1. *Spa* Sequence Analysis

The spaTyper software (http://spatyper.fortinbras.us/) was used for spa sequence analysis to determine the clonal types.

# Chapter Three
# Results

## 3.1. Identification of *S. aureus* Isolates

From each sample cultured on mannitol salt agar, 3-5 separated mannitol fermenter colonies (yellow color) were subcultured on nutrient agar and broth for further identification. All isolates showed Gram stain positive, round shape in clusters, positive for catalase, β-hemolysis on sheep blood agar and coagulase positive. Only 251 of *S. aureus* isolates were recovered from all samples, which were 136 isolates detected from bulk tank milk samples, 35 isolates from nasal human workers and 80 isolates detected from environment sources. Results showed that 83.3% (10/12) of farms had at least one BTM sample contaminated with *S. aureus*. In addition, 75% and 58.3% of farms had contaminated environments and human carrier with *S. aureus*, respectively. Data about farms and *S. aureus* isolates number in each farm are shown in Table 3.1.

**Table 3.1. Data about number of dairy cows in each farm, and number of *S. aureus* isolates recovered from each samples in each farm.**

| Farm | No. of cows | Milking process | No. of samples | | | No. of *S. aureus* isolates | | | Total of isolates** |
|---|---|---|---|---|---|---|---|---|---|
| | | | M | E | H | M | E | H | |
| **A** | 60 | MM | 8 | 3 | 2 | 26 | 4 | 0 | 30 |
| **B** | 17 | MM | 8 | 3 | 2 | 7 | 8 | 8 | 23 |
| **C** | 5 | MM | 2 | 2 | 1 | 4 | 0 | 0 | 4 |
| **D** | 60 | MM | 3 | 3 | 1 | 3 | 0 | 4 | 7 |
| **E** | 18 | MM | 2 | 3 | 1 | 8 | 7 | 4 | 19 |
| **F** | 85 | MM | 4 | 3 | 1 | 16 | 8 | 4 | 28 |
| **G** | 60 | MM | 4 | 3 | 1 | 0 | 4 | 0 | 4 |
| **H** | 300 | MM | 2 | 3 | 1 | 4 | 0 | 0 | 4 |
| **I** | 200 | MM | 10 | 6 | 2 | 32 | 21 | 3 | 56 |
| **J** | 8 | MM | 3 | 4 | 1 | 0 | 6 | 0 | 6 |
| **K** | 80 | MM | 5 | 6 | 1 | 20 | 16 | 4 | 40 |
| **L** | 450 | MM | 6 | 6 | 2 | 16 | 6 | 8 | 30 |
| **Total** | 1343 | | 57 | 45 | 16 | 136 | 80 | 35 | 251 |

*M: BTM sample

E: Environmental sample

H: Human nasal swab

MM: Milking machine

**Each colony is considered as one isolate

## 3.2. Antibiotic Resistance

Only 68 of *S. aureus* isolates recovered from BTM samples (n=39), people (n=10) and environment sources (n=19) in contact with farm animals were tested for antibiotic resistance. Isolates recovered from BTM showed high resistant against the following antibiotics: clindamycin, nalidixic acid, tetracycline, meropenem, erythromycin and trimethoprim/sulfamethoxazole. Resistance of these isolates to these antibiotics had a range from 61.5%-94.8%. A total of 7.7% of these isolates showed resistant to ciprofloxacin. The results showed that 66.7% of *S. aureus* isolated from bulk tank milk were MRSA and resistant to cefoxitin.

According to the isolates recovered from environment sources also showed high resistant against nalidixic acid, meropenem, clindamycin, Trimethoprim/Sulfamethoxazole and tetracycline. The range of resistance against these antibiotics was 63.2%-94.7%. These isolates were more sensitive to ciprofloxacin, 5.3% of *S. aureus* isolates recovered from environmental sources were resistant to ciprofloxacin. Also, these results showed that 57.9% of these isolates were MRSA and resistant to cefoxitin. In addition, *S. aureus* isolated from workers in contact with cattle dairy farms showed high resistant against nalidixic acid, meropenem, tetracycline, clindamycin and erythromycin. Resistance of these isolates against these antibiotics had a range between 50%-80%. These isolates showed more sensitivity to amikacin, imipenem, ciprofloxacin, 10% of isolates were resistant to these antibiotics. Methicillin resistant *S. aureus* was detected in 30% of *S. aureus* isolates recovered from human nasal swabs and were resistant to cefoxitin. Profile of antibiotic resistance of 68 *S. aureus* isolates recovered from bovine BTM samples, environment sources and workers in contact with bovine dairy farms is shown in Table 3.2. Representative samples showing detection of *mecA* gene by PCR in MRSA isolates recovered from BTM samples, environment sources and nasal swab workers are shown in Figure 3.1. Results in current study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were MDR.

Results of the current study showed that 58.3% of farms had MRSA contaminated BTM samples, contaminated environment and MRSA worker

carriers. Results also showed that BTM samples, environment and workers of 4 farms (C, D, G and H) were MRSA negative. Distribution of MRSA and MSSA isolates according to the farms and source of sample is presented in Table 3.3. In current study, *Staphylococcus aureus* (MRSA and MSSA) isolates were detected in 68.4% bovine BTM samples, while MRSA only was detected in 45.6% of these samples.

**Table 3.2. Antibiotic resistance profiles of 68 *S. aureus* strains recovered from different source.**

| Antibiotic | Antibiotic resistance No. (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Milk source | | | Human source | | | Environmental source | | |
| | S | I | R | S | I | R | S | I | R |
| **DA** | 8(20.5) | 3(7.7) | 28(71.8) | 2(20) | 3(30) | 5(50) | 3(15.8) | 2(10.5) | 14(73.7) |
| **CN** | 17(43.6) | 1(2.6) | 21(53.8) | 7(70) | 1(10) | 2(20) | 10(52.6) | 4(21.1) | 5(26.3) |
| **VA** | 19(48.7) | 0(0.0) | 20(51.3) | 8(80) | 0(0) | 2(20) | 13(68.4) | 0(0.0) | 6(31.6) |
| **E** | 11(28.2) | 4(10.3) | 24(61.5) | 3(30) | 2(20) | 5(50) | 7(36.8) | 3(15.8) | 9(47.4) |
| **FOX** | 13(33.3) | - | 26(66.7) | 7(70) | - | 3(30) | 8(42.1) | - | 11(57.9) |
| **SXT** | 11(28.2) | 1(2.6) | 27(69.2) | 6(60) | 0(0) | 4(40) | 4(21.1) | 3(15.8) | 12(63.1) |
| **CIP** | 22(56.4) | 14(35.9) | 3(7.7) | 8(80) | 1(10) | 1(10) | 13(68.4) | 5(26.3) | 1(5.3) |
| **IMP** | 18(46.2) | 3(7.7) | 18(46.2) | 9(90) | 0(0) | 1(10) | 8(42.1) | 1(5.3) | 10(52.6) |
| **NA** | 1(2.6) | 1(2.6) | 37(94.8) | 1(10) | 1(10) | 8(80) | 1(5.3) | 0(0.0) | 18(94.7) |
| **MEM** | 6(15.4) | 3(7.7) | 30(76.9) | 3(30) | 0(0) | 7(70) | 3(15.8) | 0(0.0) | 16(84.2) |
| **AK** | 21(53.8) | 1(2.6) | 17(43.6) | 9(90) | 0(0) | 1(10) | 10(52.6) | 1(5.3) | 8(42.1) |
| **TE** | 8(20.5) | 1(2.6) | 30(76.9) | 4(40) | 0(0) | 6(60) | 4(21.1) | 2(10.5) | 13(68.4) |

No.: number of isolates; S: Susceptible; I: Intermediate; R: Resistant

DA: Clindamycin; CN: Gentamycin; VA: Vancomycin; E: Erythromycin; FOX: Cefoxitin; SXT: Trimethoprim/Sulfamethoxazole; CIP: Ciprofloxacin; IMP: Imipenem; NA: Nalidixic acid; MEM: Meropenem; AK: Amikacin; TE: Tetracycline.

**Table 3.3. Distribution of MRSA and MSSA isolates according to the farms and source of sample.**

| Farm* | Total (n) | *S. aureus* isolates | | | |
|---|---|---|---|---|---|
| | | MRSA isolates | | MSSA isolates | |
| | | M n (%) | H and E n (%) | M n (%) | H and E** n (%) |
| **A** | 9 | 7 (77.8) | 0 (0.0) | 1 (11.1) | 1 (11.1) |
| **B** | 7 | 2 (28.6) | 1 (14.3) | 1 (14.3) | 3 (42.8) |
| **C** | 1 | 0 (0.0) | 0 (0.0) | 1 (100) | 0 (0.0) |
| **D** | 2 | 0 (0.0) | 0 (0.0) | 1 (50) | 1 (50) |
| **E** | 5 | 1 (20) | 0 (0.0) | 1 (20) | 3 (60) |
| **F** | 7 | 4 (57.1) | 2 (28.6) | 0 (0.0) | 1(14.3) |
| **G** | 1 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (100) |
| **H** | 1 | 0 (0.0) | 0 (0.0) | 1 (100) | 0 (0.0) |
| **I** | 14 | 5 (35.7) | 3 (21.4) | 3 (21.4) | 3 (21.4) |
| **J** | 2 | 0 (0.0) | 2 (100) | 0 (0.0) | 0 (0.0) |
| **K** | 10 | 3 (30) | 3 (30) | 3 (30) | 1 (10) |
| **L** | 9 | 2 (22.2) | 1 (11.2) | 3 (33.3) | 3 (33.3) |

*A-L: Farms

**M: BTM sample; H and E: Human and Environmental sample source

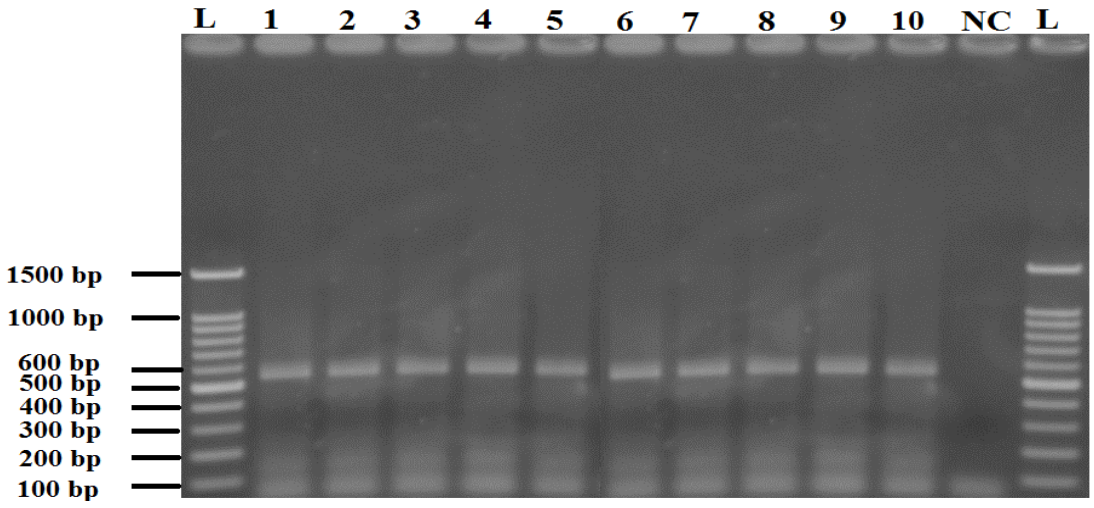


**Figure 3.1:** Representative samples showing detection of *mecA* gene by PCR in MRSA isolates recovered from different sources.

### 3.3 Detection of *spa* Type and Phylogenetic Tree

PCR product of some *spa* genes were sequences to determine the type of *spA* types circulated in cow diary farms in Palestine (Figure 3.2). Sequences of *spA* genes in this study were examined to detect repeat regions and identify the particular *spa* type using spaTyper software (http://spatyper.fortinbras.us/). Out of 18 sequences, 4 sequences which have numbers 38 (MG759487), 48 (MG759491), 30 (MG759494) and 19 (MG759504) were belonged to to *spA* type t164 and other 2 strains which have numbers 2 (MG759500) and 18 (MG759503) were belonged to t9129 and t2518, respectively (Table 3.4). Other 12 strains were nontypbeable. Three strains which have *spa* type t164 were recovered from BTM samples from different farms (I. K and F), while the fourth strain was recovered from human nasal swab who is working in farm I. Strain number 44 (MG759488) is closely related to strains which belonged to type t164, this strain has a difference in one repeated sequence. Strain number 44 (MG759488) was recovered from BTM samples from farm I. All *spa* gene sequences obtained in this study with other similar sequences of *spa* gene that retrieved from GenBank were used to construct the phylogentic tree (Figure 3.3). According to phylogenetic tree, strains number 35 (MG759497) and number 45 (MG759489) could be identical or very closed related clones, these strains 35 and 45 were recovered from BTM samples from different farms, which are farm L and farm I, respectively. According to strains number 29 (MG759493) and number 38 (MG759487) are clustered together in phylogenetic tree, even strain number 38

(MG759487) is belonged to the type t164, these strains are very closely related. These 2 strains are differed in one repeated sequence. Strains number 29 (MG759493) and number 38 (MG759487) were recovered from BTM sample and worker nasal swab from farm K and farm I, respectively.

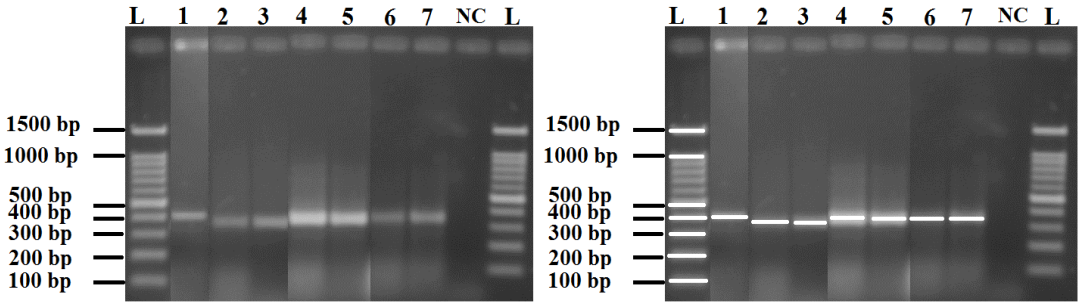


**Figure 3.2:** Representative samples showing detection *spa* gene by PCR in MRSA and MSSA isolates recovered from different sources. Lanes: L, 100 bp DNA ladder; lanes 1 to7, amplified product of *sapA* gene and NC negative control.

## Table 3.4. Characteristics of *spa*-types detected in this study.

| Strain | Start pos[1] | Repeat units[2] | Len in BP[3] | Repeat seq[4] | Ridom type[5] |
|---|---|---|---|---|---|
| 14B40 (38) | 93 | 8 | 192 | U1:G2:M1:F1:B1:B1:L1:B1<br>r07:r06:r17:r21:r34:r34:r22:r34 | t164 |
| 2A47 (44) | 160 | 8 | 192 | I2:G2:M1:C1:B1:B1:L1:B1<br>r14:r06:r17:r05:r34:r34:r22:r34 | * |
| 2B48 (45) | 84 | 8 | 192 | *:G2:M1:F1:B1:B1:L1:*<br>r569:r06:r17:r21:r34:r34:r22:r562 | * |
| 2G51 (47) | 87 | 8 | 192 | U1:J1:G1:*:*:*:Q1:B1<br>r07:r23:r12:r444:*:*:r24:r34 | * |
| 2E52 (48) | 85 | 8 | 192 | U1:G2:M1:F1:B1:B1:L1:B1<br>r07:r06:r17:r21:r34:r34:r22:r34 | t164 |
| 8A26 (27) | 89 | 4 | 96 | U1:G2:M1:A1<br>r07:r06:r17:r02 | * |
| 8C28 (29) | 97 | 8 | 192 | U1:G2:M1:F1:*:B1:L1:B1<br>r07:r06:r17:r21:r147:r34:r22:r34 | * |
| 8D29 (30) | 79 | 8 | 192 | U1:G2:M1:F1:B1:B1:L1:B1<br>r07:r06:r17:r21:r34:r34:r22:r34 | t164 |
| 9A31 (32) | 77 | 11 | 264 | U1:J1:G1:B1:B1:G1:G1:J1:A1:F1:J1<br>r07:r23:r12:r34:r34:r12:r12:r23:r02:r21:r23 | * |
| N10C35 (34) | 112 | 7 | 168 | J1:K1:F1:*:B1:L1:B1<br>r23:r16:r21:*:r34:r22:r34 | * |
| 10A36 (35) | 84 | 8 | 192 | U1:G2:M1:F1:B1:B1:*:B1<br>r07:r06:r17:r21:r34:r34:*:r34 | * |
| 10B37 (36) | 86 | 8 | 192 | U1:J1:M1:F1:*:B1:L1:B1<br>r07:r23:r17:r21:r83:r34:r22:r34 | * |

| | | | | | |
|---|---|---|---|---|---|
| 10D39 (37) | 86 | 8 | 192 | U1:G2:M1:F1:B1:B1:F1:E1<br>r07:r06:r17:r21:r34:r34:r21:r13 | * |
| N9A2 (2) | 101 | 9 | 216 | U1:K1:F1:M1:B1:B1:B1:P1:B1<br>r07:r16:r21:r17:r34:r34:r34:r33:r34 | t9129 |
| 2110 (10) | 101 | 10 | 240 | I2:M1:*:*:M1:M1:K1:A1:*:M1<br>r14:r17:*:*:r17:r17:r16:r02:*:r17 | * |
| 3015 (15) | 177 | 5 | 120 | *:*:B1:L1:E1<br>r447:*:r34:r22:r13 | * |
| 3418 (18) | 95 | 9 | 216 | T1:J1:E1:J1:N1:C1:M1:O1:R1<br>r26:r23:r13:r23:r31:r05:r17:r25:r28 | t2518 |
| 3519 (19) | 82 | 8 | 192 | U1:G2:M1:F1:B1:B1:L1:B1<br>r07:r06:r17:r21:r34:r34:r22:r34 | t164 |

[1]Starting coordinate of repeats in sequence

[2] Number of repeat units

[3] Length of entire VNTR

[4] Kreiswirth, Ridom nomenclature, but where ***** indicates a sequence that is a likely spa repeat, but does not exist in this database

[5] Ridom type name

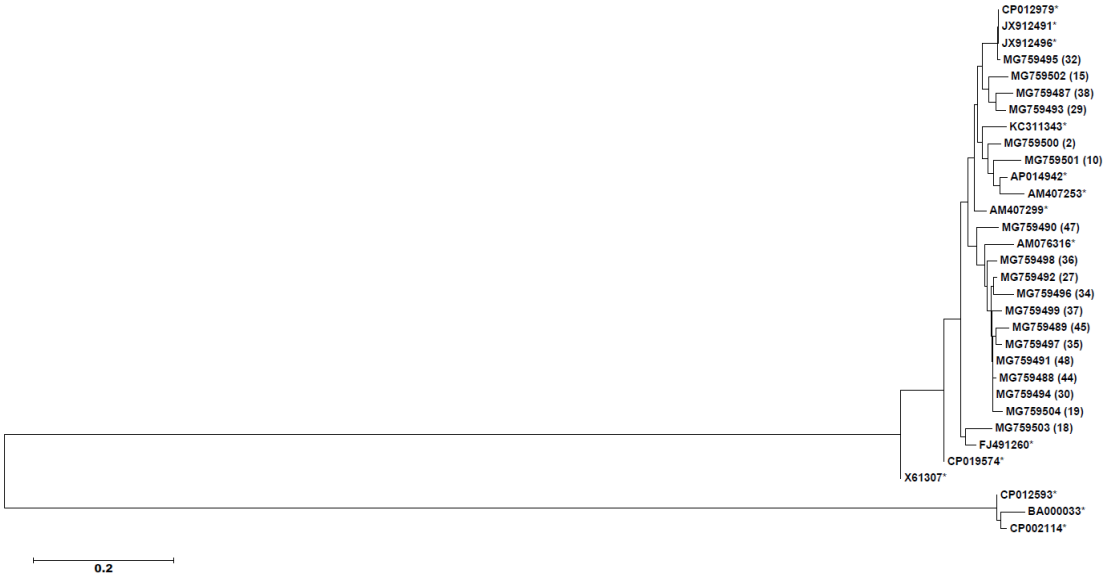


**Figure 3.3:** Phylogenetic tree constructed using the Neighbor-Joining (N-J) method based on the partial *spa* gene sequence. Reference sequences were retrieved from GenBank and denoted by asterisk. The tree was bootstrapped with 1000 resamplings, and the genetic distance corresponding is shown by the bar. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA6. Numbers between brackets near the GenBank accession numbers denoted these strains have the same numbers on dendrogram.

### 3.4. ERIC-PCR Analysis

ERIC-PCR typing of 54 *S. aureus* isolates recovered from BTM samples, environment sources of cow dairy farms and nasal swabs from workers in these cow dairy farms were clustered into 6 groups (clusters) at a 60% similarity level. Each cluster was divided into at least 2 subclusters. Profile of ERIC-PCR typing showed that there are 5 identical clones and 11 closely related clones circulating among these farms. The identical clones include C1A (isolates 11, 21, 45, 47 and 48), C4C (isolates 13, 14, 42 and 49), C2B (isolates 25 and 26), C4B (isolates 1 and 34) and (isolates 35, 36). In case C1A clone, isolate number 11 was recovered from BTM sample from farm D, isolate number 21 recovered from environment source from farm G, while isolates 45, 47 and 48 were recovered from BTM samples from farm I. On other hand, C4C clone showed that isolates 13 and 42 were environmental isolates recovered from farm E and I, respectively, while isolates 14 and 49 were recovered from worker nasal swab and BTM sample from farm E and farm I, respectively. As well as C2B clone, isolate number 25 was recovered from environment source, while isolate number 26 recovered from BTM sample from farm K. Finally, in clone subcluster C4B, which had 2 clones , the first one which had isolates number 1 and 34, these were isolated from envieonment source and BTM sample from farm A and L, respectively; the second clone which had isolates number 35 and 36 recovered from BTM samples from farm L.

In case closely related clones, subcluster C1A had isolate number 44 which is closely related to isolates number 11, 21, 45 and 48. Subcluster C1B had 2 closely related clones including isolates number 12 and 19 and isolates number 32 and 5; in subcluster C2B isolates number 24 and 28; in subcluster C2C isolates number 52 and 9; subcluster C4A had 2 closely related clones including isolates number 31 and 3 and isolate number 50 and 3 and subcluster C4B had 3 closely related clones, which were isolates number 33 and 37; isolate number 16 which is closely related to isolates number 1 and 34 and isolate number 18 is closely related to isolates number 35 and 36; in subcluster C4C isolates number 15 is closely related to isolates number 13,14,42 and 49.

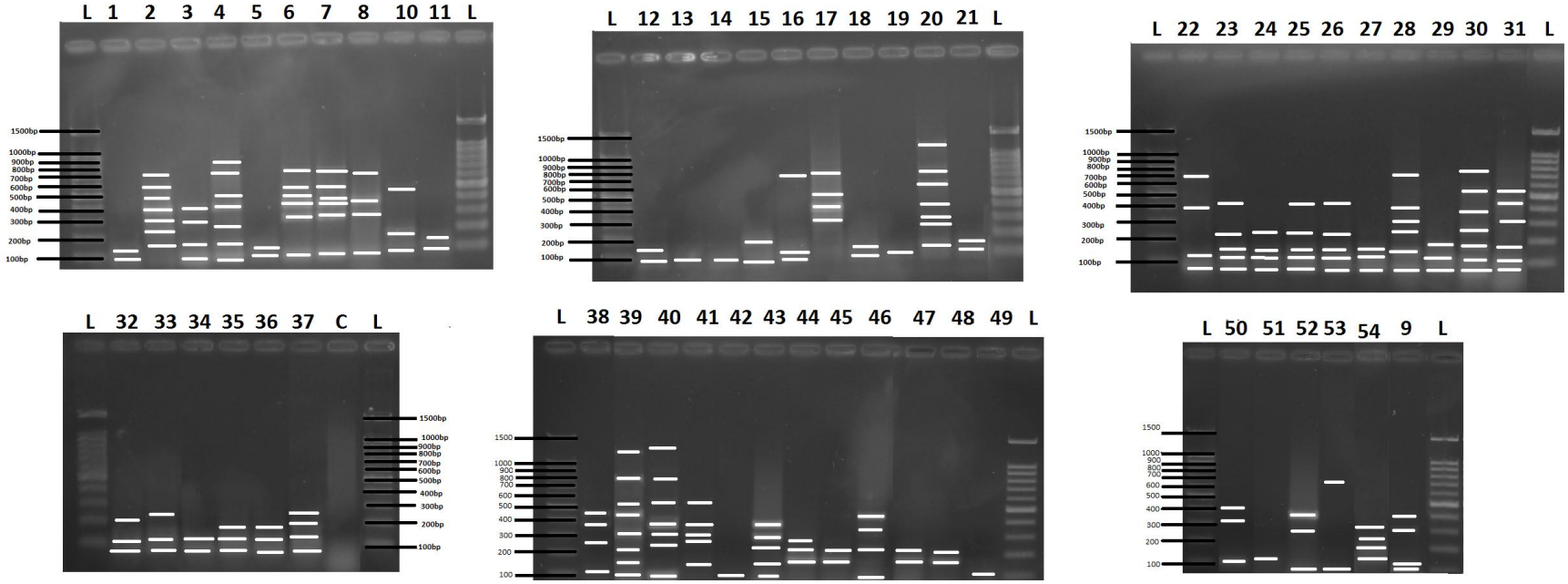


**Figure 3.4.** DNA fingerprints generated by ERIC PCR typing of 54 *S. aureus* isolates separated on 2% agarose gel. Lanes L represent the 100bp PCR-ladder; C lane: represent negative control; other lanes for ERIC PCR products.

**Farm A:** Lanes 2-9 represent BTM sample isolates; lanes 1 represent environmental isolates; **Farm B:** Lanes 51 and 52 represent BTM sample isolates; lane 50 human nasal swab isolate; **Farm D:** Lane 11 represent BTM sample isolates; lane 10 human nasal swab; **Farm E:** Lane 15 represent BTM sample isolates; lane 14 human nasal swab isolate and lanes 12 and 13 represent environmental isolates; **Farm F:** Lanes 19 and 20 represent BTM sample isolates; lane 18 human nasal swab isolate and lanes 16 and 17 represent environmental isolates;**Farm G:** Lanes 21 represent environmental isolates; **Farm I:** Lanes 43-49 represent BTM sample isolates; lane 38 human nasal swab isolate and lanes 39 – 42 represent environmental isolates; **Farm J:** Lanes 54 human nasal swab isolate and lanes 53 represent environmental isolates; **Farm K:** Lanes 26 - 31 represent BTM sample isolates; lane 22 human nasal swab isolate and lanes 23 -25 represent environmental isolates; **Farm L:** Lanes 34 - 37 represent BTM sample isolates; lane 32 and 33 human nasal swab isolate**.**

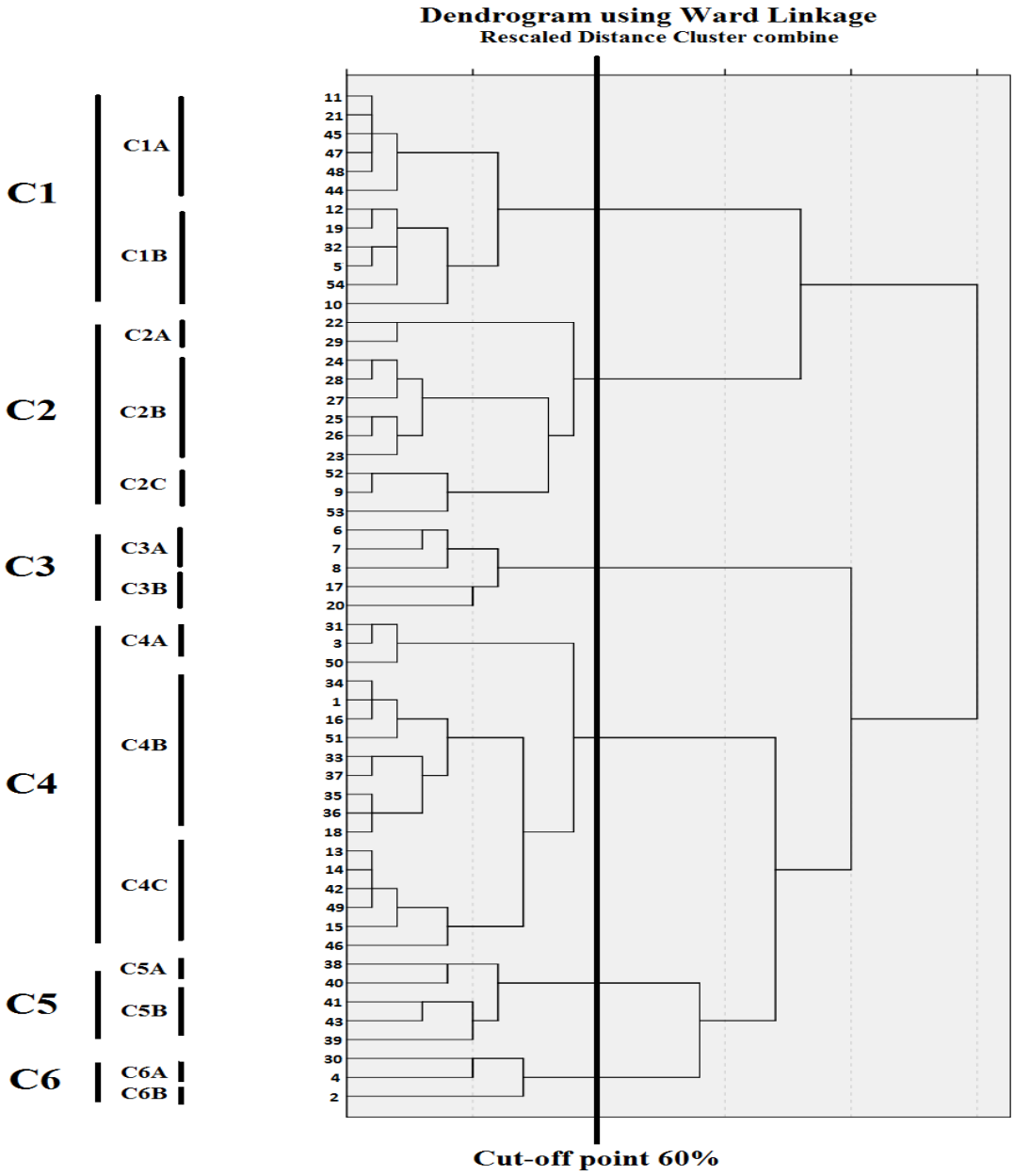


**Figure 3.5.** Dendrogram of 54 *S. aureus* isolates recovered from different sources based on the UPGMA method derived from analysis of the ERIC-PCR profiles at a 60% similarity level.
C: Cluster.

# Chapter Four
# Discussion and Conclusion

In the present time, the emergence of MRSA in cattle is considered one of the major public health problem. This type of pathogen has ability to contaminate animal origin products and can cause infection to humans as well as animals. It has been reported that these pathogens have ability to transmit from farm animals such as pigs, horses and cattle to the human workers in close contact with these animals (Caruso *et al*., 2016).

In the present study the prevalence of MRSA in cow BTM samples from cow diary farms in northern Palestine was evaluated. Furthermore, environmental samples from animal farms and nasal swabs from human workers in close contact with these animals were obtained, in order to compare the MRSA isolates recovered in humans with that found in cow BTM samples and farm environments.

Result of the current study showed high occurrence of MRSA in the cow diary farms in Northern West Bank-Palestine. It showed that 58.3% (7/12) of farms had at least one BTM sample contaminated with MRSA. This result is higher than other studies carried out previously in other countries such as in Germany and USA, which showed that the herd prevalence of MRSA had a range from 4%-16.7% (Spohr *et al*., 2011; Haran *et al*., 2012). In addition, *S. aureus* (MRSA and MSSA) in this study was detected in 68.4% bovine BTM samples. Result of this study was consistent with

previous reports from USA, Denmark and Norway, where occurrence of *S. aureus* in bovine BTM samples was 60%, 55% and 75%, respectively (Sato *et al*., 2004; Jørgensen *et al*., 2005). However, these results were in contrast to other studies from Iran, Brazil and Ethiopia, where occurrence of *S. aureus* in bovine BTM samples was 16%, 10.8% and 38%, respectively (Fagundes *et al*., 2010; Rahimi and Alian, 2013; Tolosa *et al*., 2016). In the current study, results showed that MRSA was detected in 45.6% of bovine BTM samples. This result was higher than previous studies reported from USA, Italy and Ethiopia, where occurrence of MRSA in bovine BTM was 1.3%, 3.8%, 26%, respectively (Haran *et al*., 2012; Cortimiglia *et al.*, 2016; Tolosa *et al*., 2016). The possible explanation for the high MRSA occurrence in bovine BTM and herds may be due to different reasons and conditions such as using antimicrobial agents frequently, higher levels of contact between animals during shipment, differences in farming practices such as grazing on pasture lands allow contacts between animals from different farms and may promote the spread of infection, milking machines may not be disinfected properly, high cattle population density in these farms in comparison to the small allocated area, which promotes the contact between animals and helps in the transmission of MRSA among them and most of these farms do not have special places to isolate sick or infected animals.

The results of this study demonstrate *S. aureus* recovered from BTM samples had a high level of resistance against many different antibiotics. This result was in agreement with other previous studies reported from different countries such as USA, Switzerland, Belgium, Italy, Korea, Turkey and India (Sato *et al*., 2004; Haran *et al*., 2012; Huber *et a*l., 2010; Vanderhaeghen *et al*., 2010; Cortimiglia *et al.*, 2016; Kumar *et al*., 2011; Nam *et al.*, 2011; Turkyilmaz *et al*., 2010). Results in current study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were MDR. These results were in consistent with studies previously published from Italy and Turkey, which have prevalence of MDR 90.6% and 100%, respectively (Cortimiglia *et al.*, 2016;Turkyilmaz *et al*., 2010 ). On the other hand, these results are in contrast to a report published previously from USA, which had MDR prevalence of 5.4% (Haran *et al*., 2012). This high level of resistance against many different antibiotics as well as high level of MDR may be due to the use of antimicrobial agents on dairy farms as preventive measure during dry cow therapy as well as used in food animal production systems as additives (Piddock, 1996).

In this study, ERIC-PCR profile and *spa* typing showed that some strains of the same clone had been isolated from different sample sources and different farms. For example, three strains, which have spA type t164 were recovered from BTM samples from different farms (I. K and F), while the

fourth strain was recovered from human nasal swab who is working in farm I. Strain number 44 (MG759488) is closely related to strains, which belonged to type t164, this strain has a difference in one repeated sequence and this may be due to mutation in that repeated sequence. According to phylogenetic tree, strains number 29 (MG759493) and number 38 (MG759487) are clustered together, even strain number 38 (MG759487) is belonged to the type t164, these strains are very closely related and are differed in one repeated sequence, this may be due to mutation in that repeated sequence. Strains number 29 (MG759493) and number 38 (MG759487) were recovered from BTM sample and worker nasal swab from farm K and farm I, respectively. According to ERIC-PCR profiles, there were 5 identical clones and 11 closely related clones circulated in BTM samples, environmental sources and nasal swab of workers in these farms. The presence of MRSA in both bovine milk and dairy farm environments plays a major potential risk to workers who are close contacts with cattles, veterinarians and animals that are exposed to infected cattle (Lee, 2003; Juhász-Kaszanyitzky *et al.*, 2007). This evidence suggested that these strains of the same clone or *spa* type could be circulated between cattle and humans, the transmission of this *spa* type between 2 hosts (humans and cattle) may be due to adaptation. This result was in agreement with other previous studies, which showed that some strains

were circulated between humans and cattle (Hata *et al.*, 2010; Turkyilmaz *et al*., 2010; Basanisi *et al*., 2017).

This study characterized *S. aureus* isolated from BTM samples, environmental sources and human workers in close contact with these bovine dairy farms. Results of this study showed that Palestine BTM samples is a common source of MRSA. Special attention to basic hygiene, good husbandry and biosecurity measures on bovine dairy farms have a tendency to reduce the spread of MRSA among these animals. Careful monitoring of the resistance status of *S. aureus* in cattle, humans and dairy environments is required due to some clones that circulate between them.

# Chapter five
# Recommendation

The following recommendations for this research are based on the study findings:

1. Microbiological investigations of milk quality in cattle should be performed on a routine basis, including susceptibility testing, for the screening of MRSA and MDR pathogens.

2. More stringent control measures against *S. aureus* in cattle are advisable, with severe decision criteria on animals positive for MRSA.

3. Special attention should be paid to all management practices capable of minimizing the risks of MRSA colonization, infection and transmission in the intensive dairy cattle farming system.

4. Wider surveillance should be included more farms for cattle and other animals such as goats, sheep and poultry.

5. Future studies are also needed to help identify critical areas that allow for contamination and spread within the farm environment.

6. Personnel are important sources or carriers of *S. aureus* on dairy farms, and thus hand washing and changing into clean clothing should be enforced as routine control measures.

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**جامعة النجاح الوطنية**

**كلية الدراسات العليا**

# انتشار وتوصيف المكورات العنقودية الذهبية المعزولة من حليب الصهاريج في مزارع البقر في الضفة الغربية – فلسطين

**إعداد**

**هيا إسعيد**

**إشراف**

**الدكتور غالب عدوان**

**قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.**

**2018**

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## الملخص

يعتبر ظهور المكورات العنقودية الذهبية المقاومة للميثيسيلين في قطيع الأبقار هو أحد المشاكل الصحية الرئيسية. هذه الدراسة الحالية تهدف إلى توصيف وتوثيق انتشار وتوزيع المكورات العنقودية الذهبية المقاومة والحساسة للميثيسيلين في عينات الحليب المعزولة من الصهاريج، ومعدل مقاومة المضادات الحيوية كما تتضمن الدراسة أيضا التوصيف الوراثي للنسخ المستنسخة لكل منهما باستخدام (ERIC-PCR و *spa* typing ). وبالإضافة إلى ذلك، لتقييم معدل ظهور المكورات العنقودية الذهبية المقاومة للميثيسيلين في بيئة المزرعة والأشخاص العاملون في تلك المزارع لأنه سيوفر بيانات مفيدة للدراسات الوبائية.

لقد تم جمع العينات من مزارع متفرقة متواجدة في جنين شمال الضفة الغربية– فلسطين خلال شهري أيلول وتشرين الأول – 2017 . حيث أن العدد الكلي لعينات الحليب المعزولة من الصهاريج 57 عينه بينما عدد عينات البيئة هي 45 عينه أما عينات المسحات المأخوذة من أنف الأشخاص العاملين الذين على اتصال مباشر مع الحيوانات هي 16عينه.

تم استخلاص 251 عزله من المكورات العنقودية الذهبية من جميع العينات، حيث كانت 136 عزله من عينات حليب الصهاريج و 35 عزله من أنف العاملين و 80 عزلة من البيئة المحيطة في الحيوانات.

تم اخذ 68 عزلة من المكورات العنقودية الذهبية و ذلك لدراسة مقاومتها لمضادات الحيوية منها 39 عزلة من حليب الصهاريج و 10 عزلة من العاملين في مزارع الأبقار التي تم الدراسة عليها

و19 عزلة من البيئة. نتيجة ذلك كانت 58.3% أي أن 7 مزارع من أصل 12 مزرعة تواجد فيها المكورات العنقودية الذهبية المقاومة للميثيسيلين.

علاوة على هذه فان هذه الدراسة بينت أن نسبة ظهور المكورات العنقودية الذهبية في عينات حليب الصهاريج هي 68.4% بينما نسبة المكورات العنقودية الذهبية المقاومة للميثيسيلين هي45.6% في عينات حليب الصهاريج. نتائج هذا البحث بينت أن المكورات العنقودية الذهبية المعزولة من مصادر مختلفة هي على درجة عالية من المقاومة لمعظم المضادات الحيوية المستخدمة في هذا البحث. كانت نسبة المقاومة المتعددة للمضادات الحيوية (MDR)، 100% و 69.2% لعزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين و الحساسة للميثيسيلين، بالتتابع،المعزولة من عينات حليب الصهاريج.

أن نتائج التي تم الحصول عليها من ERIC-PCR و *spa* typing توضح بعض السلالات المعزولة من مصادر مختلفة ومزارع مختلفة هي تابعه إلى نفس السلالة. على سبيل المثال أن (*spa* type t164) هي عزله مشتركة بين عزلات صهاريج الحليب و مسحات من أنف العاملين من مزارع مختلفة .هذه النتائج تؤكد مدى قدرة المكورات العنقودية للانتقال من الإنسان إلى الأبقار ولهذا سيتطلب الحذر والانتباه لنظافة والتعقيم بطرق الفعالة للتقليل من انتشار مثل هذه السلالات.