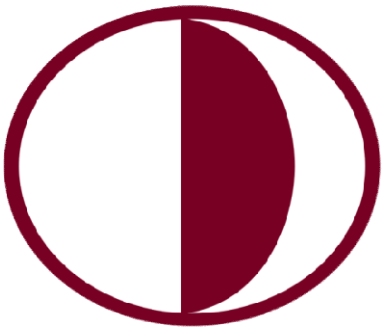

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Investigation of Biofilm Formation of *Staphylococcus aureus* Isolates

MOATAZ SHAIRA

MASTERS THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY
DEPARTMENT

MENTOR

PROF. DR. TAMER SANLIDAG

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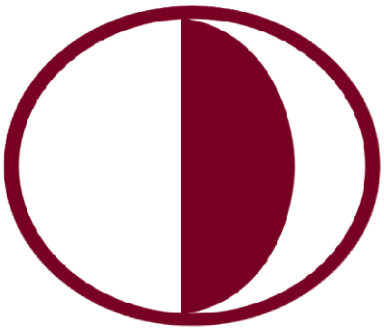

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APPROVAL

The Directorate of Health Sciences Institute,

This study has been accepted by the Thesis Committee of Medical Microbiology and Clinical Microbiology Department as Master's Thesis.

The Thesis Committee:

Chair of the Committee: .....

Mentor:Prof. Dr. Tamer Sanlidag

Members:Assist.Prof.AyseArikan

Prof.Dr. Murat Sayan

Approval:

According to the relevant articles of the Near East University Postgraduate study education and examination regulations; this thesis has been approved by the above mentioned members of the Thesis Committee and the decision by the Board of Directorate of the Institute.

Prof. Dr. Kemal Hüsnü Can Başer

Director of the Institute of Health Sciences

STATEMENT(DECLARATION)

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all theinformation in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

Name, Last name:Moataz, SHAIRA
Signature:
Date:

DEDICATION

To my father Yousef, my mother ,Amera and my children Yousef and Amera .

ACKNOWLEGMENTS

I am highly grateful to my supervisory team;Prof. Dr. Tamer Sanlidag.I deeply appreciate Dr.FerdiyeTaner for the training, encouragement, and patience fostered during the course of this research. I would also like to thank to muchAssist. Prof.AyseArikan for her guidance.

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Finally, I appreciate my parents and other members of the family for the love and prayers.

CONTENT

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ABBREVIATIONS AND SYMBOLS

CWA	Cell Wall-anchored
CRA	Congo Red Agar
CDC	Centre for Disease Control
EPS	Extracellular Polysaccharides
MRSA	Methicillin Resistant *Staphylococcus aureus*
SAB	*S. aureus*bacteraemia
SSTIs	Skin and Soft Tissue Infections
TM	Tube Method
VISA	Vancomycin-Intermediate *Staphylococcus aureus* (VISA)
VRSA	Vancomycin-resistant *Staphylococcus aureus* (VRSA)

***Staphylococcusaureus*izolatlarınınbiyofilm oluşturma özelliklerininaraştırılması**

ÖğrenciAdı:MoatazShaira

Danışmanı:Prof. Dr. Tamer Şanlıdağ

AnabilimDalı:TıbbiMikrobiyolojiveKlinikMikrobiyoloji

1. ÖZET

Amaç: Bu tezinamacıönemlibirinsanpatojeniolan*Staphylococcus aureus*izolatlarıtarafındanbiyofilmoluşumunun invitro olarakgösterilmesidir.

GereçveYöntem: YakınDoğuÜniversitesiMikrobiyolojiLaboratuvarınagönderilen (kan , idrar, deri vs.) numunelerdenizoleedilen 38 *Staphylococcus aureus*izolatıçalışmayadahiledildi.

Stafilokokizolatlarınınbiyofilmoluşturmaözelliklerifenotipikvemikroskobikolarakdeğerlendirildi.

BulgularveSonuçlar: Eldeedilensonuçlar*Staphylococcus aureus*izolatlarınınbiyofilmoluşturmaözelliğinidoğrulamaktadır. Bu çalışmaileayrıcaglikozvesukrozşekerlerininbiyofilmoluşumuüzerindeetkiliolduğugösterildi.Sonuçolarakbuçalışmaile*Staphylococcus aureus'un*biyofilmoluşturmayeteneğinesahipolduğuveuygulananikiyöntemkullanılarakkolaycatespitedilebileceğigösterilmiştir.

AnahtarSözcükler:*Staphylococcus aureu*s, biyofilm,gram pozitifbakteri,enfeksiyon, hastane

Investigation of biofilm formation of *Staphylococcus aureus*isolates

Student Name:MoatazShaira

Mentor: Prof. Dr. Tamer Sanlidag

Department: Medical Microbiology and Clinical Microbiology

1. SUMMARY

Aim: The thesis aimed to study in vitro detection ofbiofilmformation by *Staphylococcusaureus*which is an important human pathogen.This investigation was performed due to increase infection caused by *S. aureus*and being recalcitrant to treatment.

Material and Method: A total of 38samples (blood, skin and urine) were collected from patients in Near East University Hospital. These samples were used to isolate *S. aureus*strains. Biofilms formation was evaluated using two methods; culture method and microscopy.

Findings and Results: The results and biofilm formation obtained from this study were confirmed by *S. aureus*isolates by using two different methods.Furthermore, this study also confirmed the role of glucose and sucrose in enhancing biofilm formation. Conclusively, it was shown that *S. aureus*isolates have the ability to form biofilm and can be easily detected using two methods.

Key Words: *Staphylococcusaureus*, biofilm, gram-positive bacteria, infections, hospital.

1. INTRODUCTION

This chapter gives the overall introduction to the organism; *Staphylococcus aureus,* its biology, pathogenesis, diseases it causes, biofilm formation and constituent, the aim of the study and objectives, as well as significance of the study.

1.1 Background

*Staphylococcus aureus*is a gram-positive bacterium that usually colonizes humans and other animals including warmed blooded ones. The morphological characteristic of this organism includethe following; ability to grow on most bacteriological media, and more rapidly at 37°C with smooth, round, raised and glistening colonies (Mootz, 2013). A large number of human population is being colonized by this organism at different sites of the body. Mootz (2013) reported that significant number of humans (25%) are colonized by *S. aureus*, and this organism is known to cause infections in healthcare as well as in community (Davis *et.al.,* 2007; Klein et.al., 2007).The colonization is usually found in areas such as anterior nares, and the throat, skin, axilla, perirectal area, and groin are high risk areas. It is considered a human commensal but acts as an opportunistic pathogen and its carriage is linked with increased risk of further infections. In an extensive review, Tong *et al.,* (2015) estimated that 10 to 30 per 100,000 person-years have health implication with *S. aureus* in industrialized world and this increased medical cost to the global health sector by the infection of this important organism, which leads to increase hospital bill and stay, increase in budgets of health ministry by government. The incidence of *S. aureus*bacteraemia (SAB) varies among the different parts of the world, for instance in developed world, it is up-to 30 in every 100, 000 persons, and this number is much more in developing countries that are characterized with poor monitoring and control measures of *S. aureus* associated infections. The incidences of pneumonia and bacteraemia mostly caused*S. aureus* infections and it consumed greater total budget of medical cost (60%), in addition it results to substantial number of deaths. Amongst the infection that cause long term hospitalization is endocarditis,

around 26 days and by implication, result to increase in medical cost per patients. Similarly, infections due to surgical operations can result to hospitalization of about 14 days with significant economic burden. Globally, large numbers of health humans (25-35%) are known to be carriers of *S. aureus*which can be found on the skin or mucous membranes. On the current world's population, more than 2 billion people can be said to have carry the organism, based on the estimation of Dutch and America, greater number of this data harbour *S.aureus* infection, however, some of the organisms have already developed resistance to the available agents, as a result, they are called Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Deurenberg, et al., 2007).

Similar trends of increasing infection with *S. aureus* is associated with infective endocarditis (IE) with a figure from 1970-2010the study was conducted in Europe and America, and the result showed IE cases is between 16-34% and no change was observed in all the cases over the years (Tong et al., 2015). Other diseases associated with *S. aureus* include pneumonia, which is usually implicates individuals with preexisitingdiseases especially lung disease that are on ventilators, those with bone disease such as osteomyelitis, which can be resulted due to the dissemination of*S. aureus* through bloodstream (CDC,2011). *S. aureus* causes different disease conditions of skin and soft tissue, from simple such as impetigo and uncomplicated cellulitis to the fatal disease condition. It is the most common pathogen isolated from surgical site infections (SSIs), cutaneous abscesses, and purulent cellulitis but rarely caused urinary tract infections (Deleoet al.,2010). The severe health complication resulted by this pathogen is a consequence of its ability to become resistant to certain antibiotics, certain *S. aureus* are therefore referred to as MRSA, *Vancomycin-intermediate Staphylococcus aureus* (VISA), and *Vancomycin-resistant Staphylococcus aureus* (VRSA) and of recent concern is its association with biofilm formation which further complicates many diseases conditions (CDC, 2011).

Looking at the population at risk of *S. aureus* infections, anyone can develop a *Staphylococcus species*infection;however, the chance is higher in certain individual than others, for example in chronic diseases such as cancer, lower respiratory diseases, eczema etc. In a hospital environment, the prone to the infection is higher

than other environment, and amongst the immunocompromised individuals and those undergoes intravenous procedures. It is evident that duration of hospitalizationcontributes to the infection with MRSA and such can increase the chance of infections with multidrug resistant organisms. By this, the risk of transmission will increase among patients and hospital personals. Thus, the pathogen or its resistant variants can be spread by the fact that it is found on skin,and also molecular research indicated that the spread is clonally based in hospital.Every hospital has unique sets of clones spreading at a particular period (Safdar and Maki, 2002).

MRSA are not only confined to the hospital setting but have also been reported amongst communities.People who are prone to infections are usually those found in a closed environment such as prisoners, during physical activity and children have been described with MRSA infections acquired in the community (Salgado et. al., 2003). Similarly, Moran et al., (2005) reported the increase in community-associated MRSA infections (CA-MRSA) and outbreaks among high risk individuals in inmates, IV drug abusers, athletes, and those with sexual orientations such as men who have sex with men. CA-MRSA has been reported in diseases such as infection in skin and other soft tissue, and also age dependant such as paediatrics (Bancroft et. al., 2003). There is increase in trend of MRSA pathogens isolated in community in comparison with those originated from hospital (Naimi et al., 2003; Moran et al., 2005).

1.2. *Staphylococcus aureus* biofilms

By definition, a biofilm is a complex community of sessile microenvironment that contain cells which are attached to each other. The nature of the cells will form a matrix and form what is known as microbial mats in a polymatrix extracellular constituents and show modified phenotype in terms of their genes, nature of growth and production of proteins (Archer *et. al.,* 2011). In other words, a biofilm is defined as a sessile microbial community in which cells are attached to a surface or to each other cells and covered by a protective extracellular polymeric matrix. This type of growth shows changed physiology of the bacteria in term of its gene expression and production of molecules such as proteins.

There is increasing attention on studies for better understanding of bacterial biofilms and how its growth nature is related to human diseases.The thickness of biofilm varies from a single cell layer to a multicellular community covered by a viscous polymeric layer and also from homogenous species to heterogenous microbial community (Donlanet. al., 2002). It is established that a biofilm can comefrom a one cell, it can be influenced by the different environmental conditions in the whole community can potentiate the development of distinct subpopulations. The concentrations of oxygen, nutrients and electron acceptors can result to the formation of multiple genes biofilm community (Rani et al., 2007). Foster et al.,(2014) described the stages of biofilm development into three broad events: initial attachment, biofilm maturation, and dispersal. Firstly, the attachment of single planktonic species will bind in a reversible manner, and this will further bind together irreversibly if the initial attachment is not separated. The attachment is made possible by surface proteins known as microbial surface constituents recognizing the matrix molecules that are adhesive in nature.

S. aureus has the ability to attachdifferent surfaces including medical devices as well as living tissues, which will further mature and form persisters in chronic diseases conditions. The formation of a biofilm will provide additional advantage to the cells such as formation of resistance to drugs due to reduced penetration to the cells, resisting immune systemthereforetreatment of such infection will be difficult. (Lister and Horswill, 2014). The matrix of *S. aureus* biofilm is complex that is firmly attached to cells in the mature structure, however, other factors play significant role in the formation of the matured biofilm such as host factor, proteins and as well as other biomolecules such as polysaccharides and DNA. Each of these factors play different role and contribute separately depending on the nature of the bacterial strains and environmental factors (Fitzpatrick et al., 2005; Abraham and Jefferson, 2012).

1.3. Biofilm formation and constituents

As complex consortium of microorganisms can easily develope biofilm regardless of the material provided certain conditions are met, such conditions include availability of nutrients, proper surface area, such as rough surface or any

surface that would allow easy attachment. It is worthy to note that physical conditions play role in the attachment process, for instance, the charges nature of the surface; hydrophobicity and the rate of flow facilitate the biofilm formation. (Donlan, 2002). Coming together of molecules of organic molecules and inorganic constituents facilitate the quick formation of a coated layer on a surface in contact with an aqueous fluid. This results to the formation of conditioning film which is an important step in the formation of biofilms, because it modifies the properties of the surface so that microbial adhesion becomes possible by Van der Waals attraction forces and electrostatic charges(Tarver, 2009).

Agle, (2007) described the steps of biofilm development as complex process. The process begins with the attachment of separate cells to the coated film in a dynamic and reversible nature; at this stage it is stilla mild attachment and can be removed easily. In the subsequent stage, the cells produce polymeric substances that allow strong binding of many cells in a manner that depicts three-dimensional structure of extracellular polysaccharide (EPS) and other biomolecules such as nucleic acids, proteins, fatty acids and water whereby the microorganisms make firm clusters (Fig 1). This stage is difficult to be reversed, and very difficult to remove because the biofilm has been already established.At the final stage, the biofilm starts to disperse cells so that another cycle will commenced in another surface (Dolan, 2002).

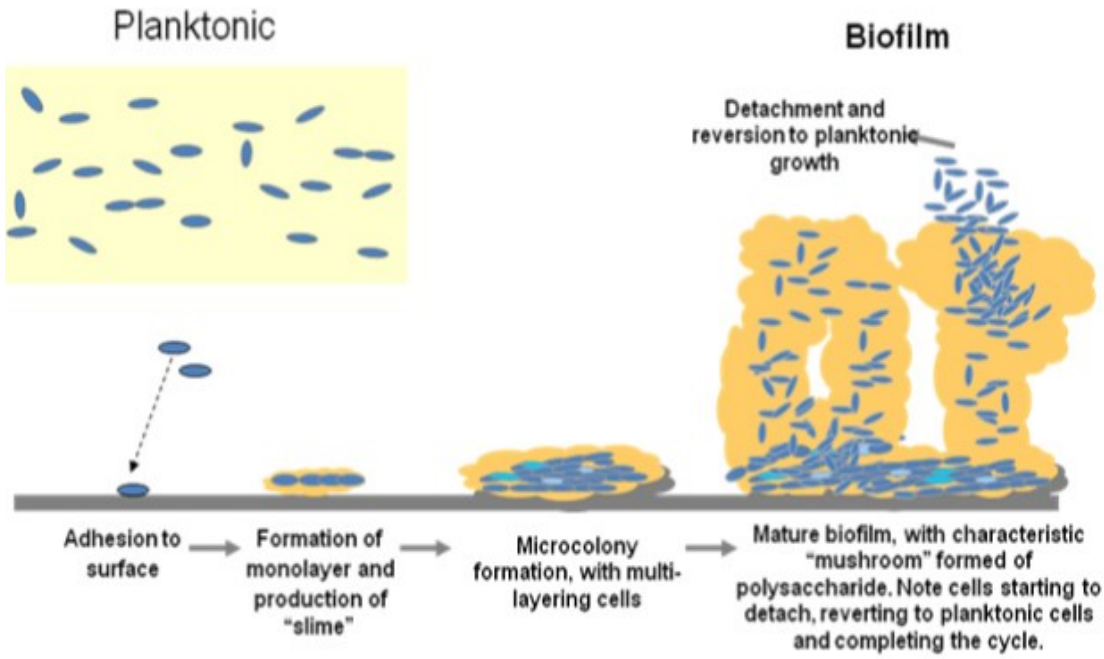

Fig 1. Biofilm formation step (Adapted from Mortensen, 2014)

Vast number ofgram positive and gramnegative bacteria, yeast, and fungi can form biofilm, although some can easily form than other species by their ability to make structures such as extracellular bacterial organelles for instance fimbriae, pili flagella and any organelle that facilitates attachments. A single microbial species may form a homogenous biofilm, but most biofilms are mixtures of different species, resulting to a heterogeneous biofilm(Bactoforce, 2013). To create an enabling environment, there exist channels of communication between bacterial species through diffusible molecular signals which control the gene expression, by a condition called quorum sensing. This madethe interplay among the cells mutually feasible in the whole biofilmcommunity by coordinating thesupply of essential requirements for the survival, such as water and nutrients to the individual cells andthe removal of waste products. This association helps the cells to be more resistant and more stable to the hostile environments and stress (Dolan, 2002).

1.4. Aim and objectives of the study

- To detect biofilm formation by *Staphylococcus species*.
- Using different methods to detect biofilm formation. Main interest is detecting extracellularpolysaccharide (EPS) production, as the major component of biofilm.

1.5. Significance of the study

This thesis will provide information about two methods that can be used to detect biofilm production amongst *Staphylococcus* species.

2. GENERAL INFORMATION

Staphylococcus aureus colonize human population of about 25% and certain percentage (75%) are intermittently or not colonized (Ray et al., 2012). The colonization is well known agent responsible for causing chronic biofilm infections. As a gram-positive bacteriathe organisms mostly colonizes humans and other warm-blooded.Sitesof colonization are usually the skin, throat, nose, and groins regions. These areas are believed to have selective pressures that favour the growth and colonization of the organism (Micheal, 2013). *S. aureus* is a human commensal butacts as an opportunistic pathogen and harbouring such organism increase the chance of infection in future. *S. aureus* is ranked as one of the most common agent of healthcare and community-associated infections (Mootz, 2013).

Healthcare associated infections increasedamongst the patients with existing high risk factors such as immunosuppression or those with implants. These infections are usuallybacteraemia and mainly results from *S. aureus* transmission by hospital personals that are colonized partly (Tong et al., 2015). Antibiotic resistance has been a great source of concern in the treatment of *S. aureus* infections, because the key agent in the treatments is becoming less effective due to the formation of biofilm(Micheal, 2013). Historically, the resistant form of *S. aureus* infections first became health issue in the last 50 years, when the organism became resistance to

penicillin, an agent that previously used to treat it successfully.The emergence of the resistance was made possible by acquiring a plasmid that encoded enzymeswhich can break down the drug known as beta-lactamase. Over the years, MRSA were reported globally, which contribute to significant mortality and morbidity (Deurenberget al., 2007). Furthermore, the infection related to MRSA has spread outside healthcare settings, thus led to emergence of CA-MRSA. The strains are mainly associated with skin and soft tissue infections, and also cause invasive disease condition (Mootz, 2013). On comparison, these strains are more transmissible andhypervirulent to MRSA related to healthcare settings. It is observed that the virulence of CA-MRSA is higher in various models of infection and have capability to escape actions of neutrophils, the exactstrategy used is not wellunderstood but many hypothesis was made to contribute to the evasion of immune system, such factors include Panton-Valentine leukocidin (PVL), type 1 arginine catabolic mobile elements (ACME), and the high levels of *agr*-regulated virulence factors (Li et al., 2009).

2.1*Staphylococcus aureus* pathogenesis

S. aureus results to number of disease conditions ranging from acute to chronic infection. The infections of skin to other tissues and to life threatening invasive conditions such as bacteraemia, bone diseases, heart related infections to pneumonia (Balenet al.,2013). The organism employs diverse sets of virulence factors to cause diseases; these factors include ability to secrete proteins and adhesions, and also structural associated virulence factors such as collagen binding proteins and plumbing factors A and B (Mootz, 2013). Furthermore, Mootz (2013) described the structural associated virulence factors of the organism to have been linked to cell wall peptidoglycan in a covalent manner by the enzymes known assortase A and collectively mediate attachment to host matrix proteins such as collagen, fibronectin and fibrinogen. Elbarasi (2014) extensively described the virulence mechanism use by the organism to escape immune system of the host which subsequently result to diseases.

Lei et al., (2012) describedthatthis pathogen is ableto infect many tissues in human body due to the expression of vast mechanisms that aid the pathogenesis of the organisms.These include surface associated proteins, toxins, and enzymes such as

proteases, which help to overcome the host immune mechanisms. It is worthy to note that the virulence factors are expressed in a coordinated manner which involved some regulators such as trans-acting global regulators, alternative sigma factors, and small non-coding RNAs.

The strains of CA-MRSA usually cause infections in a confined site, simply by the actions of many enzymes, such as protease, lipase, and hyaluronidase that destroy the tissues in immune-suppressed people. These strains exhibit increased virulence resulting to serious infections even in healthy person; however, their role in the pathogenesis of disease is still poorly understood (Elbarasi,2014).

2.2.*Staphylococcus aureus*biofilm formation

*S. aureus*can cling to surfaces and results in the establishment of a chronic disease. Studies indicate that *S. aureus* can form biofilm on host surfaces such as heart valves, bones, cartilage, and medical device such as catheters and orthopaedic devices (Ahn et al., 2008; Kiedrowski et al., *2011*). Similarly, the organism also has ability to form biofilm on inanimate objects such as pipes or any foreign body objects (Fey and Olson, 2010). This is also similar withother species of Staphylococcus such as*S.epidermidis* which is one of important nosocomial pathogen causing infections associated with indwelling biomaterials as indicated by study of Fey and Olson (2010).This assertion wasalso confirmed by studiesof Kırmusaoğlu (2016).The author described these two species of *Staphylococcus* as the commonest cause of device-associated indwelling infections.The nosocomial and community acquired infections can produce biofilm as a virulence factor. Furthermore, Kırmusaoğlu described biofilm infections caused by *S. epidermidis*and *S. aureus* are particular threat in hospitalized individuals and those with impaired immunity across the world due to their tough and reduced susceptibility to the key agents that used to treat them. Staphylococcus species use biofilm in pathogenesis due to the fact that biofilm has ability to strive regardless of the stress conditions which can be metal toxicity, ultraviolet damage, oxygen deficient conditions, acid exposure, saline

condition, pH gradient, bacteriophages, antimicrobial actions and host immune defence system (Romling and Balsalobre, 2012).

In an extensive review of Archer et al., (2011), indicated that*S.aureus*biofilm mode of growth is highly regulated by complex genetic factors which contribute to the ineffectiveness of the host immune reaction towards persistent biofilm infections, hence result to chronic disease. However, biofilm formation is not a criterion for persistent infections rather considered as a major contributing factorfor pathogenesis if notremove or treated on time. Many studies show that *S. aureus* can form a multi-layered biofilm embedded within a glycocalyx or slime layer with heterogenous protein expression throughout. First studies revealed the composition of the glycocalyx as mainly contain teichoic acid as about 80% and other components include the Staphylococcaland the host proteins (Archer et al., 2011).

Recently, the details of the component of biofilm revealed the specific polysaccharide antigens known as polysaccharide intracellular antigens (PIA) which contained b-1,6-linked N-acetylglucosamineresidues (80–85%) and less concentrations of an anionic fraction of non-N-acetylated D-glucosaminyl residues that consist of phosphate and ester-linked succinct amount to 15–20% (Fitzpatrick et al., 2005).The process of biofilm production is a complex and multifaceted process but generally can be categorized into the following steps (phase); Attachment (adhesion or adherence) phase,accumulation phase and the detachment phase. The stages are demonstrated in (fig 2).

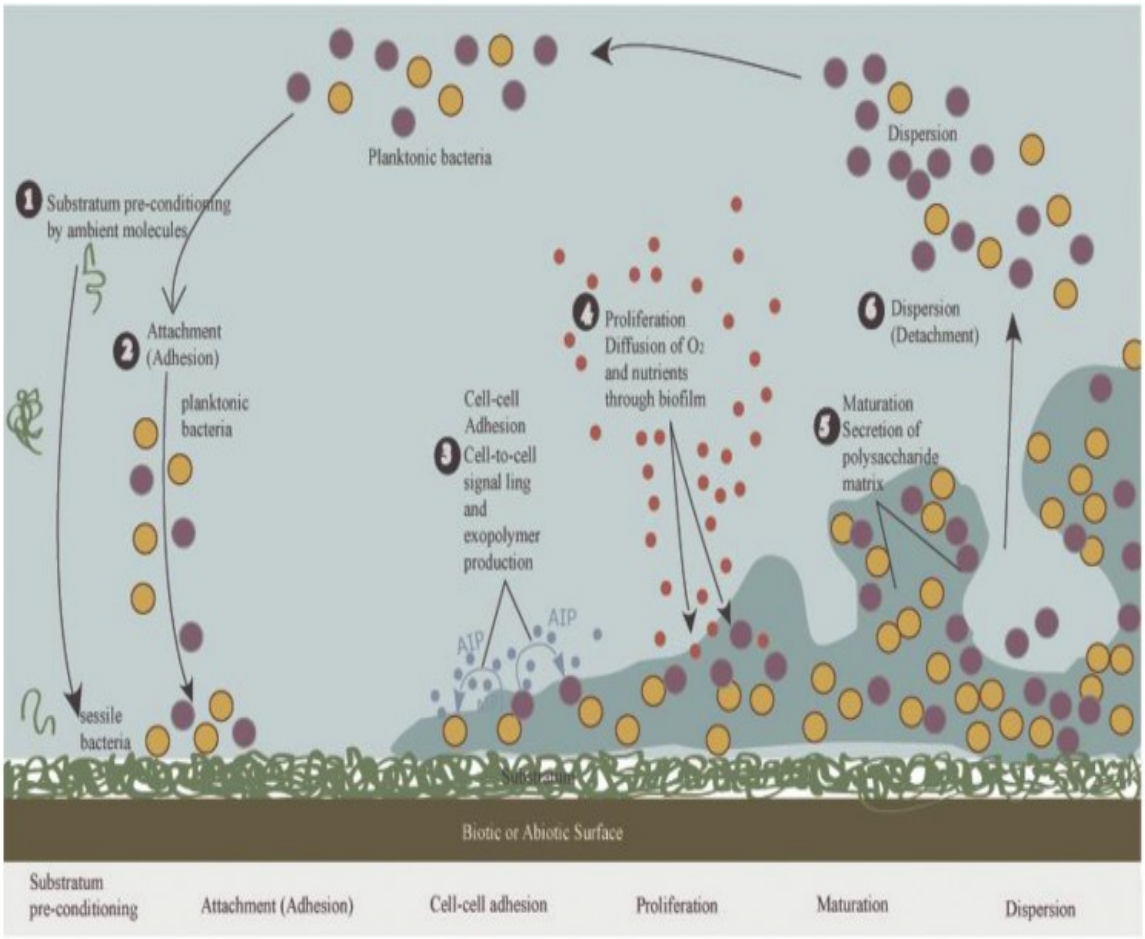

Fig 2. The stages of biofilm formation (Adapted from Kırmusaoğlu, 2016)

2.2.1 Attachment (adhesion or adherence) phase

Once the criteria that favour biofilm establishment are reached, biofilm formation commences with the initial adherence of the organism to a surface that act as a substrate for microbial adhesion which further aggregation end up in formation of cell–cell adhesion as indicated in (fig2).

Studies indicated that the adherence of Staphylococcal to nonliving abiotic surface of indwelling medical devices such as prosthetic device is mainlydepends on the physical and chemical structure of the materials.On the other hand, the structural parts of the Staphylococci such as teichoic acid, lipoteichoic acid, accumulation-associated proteins (AAP), autolysin AtlA and AtlEattacht to living surfaces.Staphylococcal adherence is achieved by the means of cell wall-anchored

proteins, for instance fibrinogen-binding protein SdrG/Fbe of *S.epidermidis* and fibrinogen-/fibronectin-binding proteins FnBPA,FnBPB and clumping factors A and B of *S. aureus* (Rupp, et.al., 2001; Houston, et al., 2001; Conlon etal., 2014). Furthermore, Zotolla and Sasahara (1994) demonstrated that for biofilm formation to begin, the populationof the cells need to be around 10^6 to 10^7colony forming unit / cm2 for proper adhesion and that value lower than those would just be an indication of an adhesion process.

2.2.2. Accumulation (aggregation or maturation) phase

At thefirst step of biofilm development; Staphylococcus speciesadhere to surfaces of both living and nonliving and produce EPS with the help of ica operon which is an extracellular matrix (ECM) developed by PIA/PNAG, extracellular DNA (eDNA), and surface proteins [cell wall-anchored (CWA) proteins] in ica independent form, and bacterial colonies become mature (Kırmusaoğlu, 2016). To provide further adherence, cell wall-anchored (CWA) protein facilitates intracellular adhesion as well as helping biofilm accumulation and maturation (Spezialeet al., 2014). At this stage, the biosynthesis of polysaccharide intracellular adhesion/poly-N—acetylglucosamine is formed in cell clusters until multi-layered structure biofilm is achieved. Staphylococcalspecies use their surface proteins such as clumping factors A and B, fibrinogen/fibronectin-binding proteins FnbA and FnbB of the pathogenor the fibrinogen-binding protein SdrG/Fbe of another species,*S. epidermidis* that are cell wall-anchored protein (CWA) also aid intracellular attachment and produce the aggregation of bacteria in ica-independent biofilm development formation rather than PIA (Foster,et. al., 2014). This indicates that even within the staphylococcal species there is difference in the used surface proteins to achieve the development of biofilm.

This makes the development of biofilm process a complex and a multi-step fashioned. Boles and Horswill(2014) revealed that the first cell-surface interaction of motile bacteria, is achieved by their flagella. Afterthat, the motile species of the bacteria will make cellular changes in biofilm by losing their motility with not functioningflagella, hence becoming non-motile.

2.2.3. The detachment (or dispersal) phase

In the last step of biofilm development; detachmentwill occur by changes from sessile cells to planktonic state that can disseminate to other surfaces and allow further colonization which yield biofilm in the new area. Microbial enzymatic activity leads to break down of the biofilm matrix by dissolving with the actions of enzymes such as proteases, nucleases and a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) acting as surfactants. At the detachment phase, gene expression occurs that characterized the specific functions such as motility by transcription of pilus and ribosomal proteins and can be noticed in planktonic cells for those that are not motile in nature. Lister and Horswill (2014) studied the enzymatic dispersal mechanism and revealed different enzymes mediated mechanism such as protease-mediated dispersal, nuclease dispersal mechanism, and dispersion B-mediated mechanism.

2.3. Types of biofilm development

During biofilm formation, two strategies are involved; PIA-dependent and PIA-independent. In the PIA-dependent biofilm formation, the production of PIA can be demonstrated in vitro from UDP-N acetylglucosamine through products of the intercellular adhesion (ica) locus. For the formation of biofilm as well as the bacterial virulence, expression of genes and other products of the ica are up-regulated in response to anaerobic growth conditions as exhibited bybiofilm environment. Under anaerobic conditions, Staphylococcal respiratory response regulator, SrrAB, will induce PIA binding of a 100 bp DNA sequence upstream of the icaADBC operon. Furthermore, there are other environmental factors which are significant in the regulation of ica, including glucose, ethanol, osmolarity, temperature and antibiotics such as tetracycline (Fitzpatrick et al., 2005).

This type of biofilm formation has been succinctly demonstrated the intracellular attachment by the actions of the charges, positive charge on PIA and negative charge on the bacterial surfaces. It is worthy to note that the*icaADBC*genesare found in all *S. aureus* strains. *Ica* locus have been reported vast numberof Staphylococcus species like *S. aureus* and *S. epidermidis* but except (*Staphylococcushaemolyticus)*and

(*Staphylococcussaprophyticus*)(Kırmusaoğlu, 2016). On the other hand, PIA-independent biofilm formation can happen in an ica-independent way. The arlRS two-component system was exhibited to repress biofilm development, and when deleted can cause the firm attachment and production of PIA. In general, Fitzpatrick et. al., (2005) described biofilm as sets of microorganisms whereby cells attach to each other on a solid surface, in a way that allow floating of microbes in liquid.

Biofilms are ubiquitous that can exist in all surfaces of biotic and abiotic, however, this can be limited especially in high places such industrial, and hospital settings. The major parts of mature biofilm are bacterial cells (25%) and significant percentage(75-95%) is glycocalyx matrix as described by previous studies (Fey and Olson 2010, Archer et. al.,2011). Kirmusaoglu (2016) also demonstrated the other type of biofilm formation is not a one way process by PIA as the major constituents of the development of biofilm, there exist other proteins.Removal of *icaADBC*can stop production of PIA and also the biofilm formation, however, the bacterial virulence is not altered. In this case, biofilm formation can be constructed asindicated in the study of in catheter infection that led to the biofilm formation of clinical isolates of *S. aureus* of which *ica*cluster is mutated. Study by Fitzpatrick et al., (2005) showed that biofilm formation of the *icaADBC* operon-deleted MRSA mutants was not altered, and in other side, the biofilm formation of the *icaADBC* operon-deleted MSSA mutants was affected.

Overall, the production of *S. aureus*biofilms and thecell-cell interactions are fastened by the action of α-toxin which is a haemolytic toxin.On the other hand, β-toxin which is a sphingomyelinase results tohaemolysis and lyse lymphocytes in turn helps insimulative role in the biofilm production of *S. aureus* by covalently cross-linking to itself in the presence of DNA in matrix of Staphylococcal biofilms (Husebyet. al., 2010).

2.4. Methods of biofilm detection

Many studies have reported the detectionof biofilm on different surfaces. Mootz et al., (2013) demonstrated biofilm formation on surfaces regardless of the coating. The authors used a method of coating microtiter plate with plasma as reported by previously researches. For the coated plates surface, it was incubated overnight at 4°C before the plasma was removed by aspiration, then followed by addition of other additives and adjustment of conditions. This technique is known as microtiter plates biofilms. Another method use in detection of biofilm is flow cell biofilms, where bacteria are supplemented in a flow chambers for certain period (48 hours) and biofilm biomass can be detected. In a study by Sarkisian (2011) which was used to categorised and quantified biofilm production in unique clinical strains of MRSA revealed that biofilm from clinical isolates of MRSA are originated from catheters.

The surface of catheters create proper conditions for biofilm growth, especially forurine. Interestingly, a small case of biofilm production was observed in MRSA isolates obtained in the nares. This indicates that for a biofilm to develop adequate surface condition needs to be achieving (Sarkisian, 2011). Furthermore, there is way of modifying the traditional method of detection as employed by Tang et. al., (2010) and also optimization of the growth conditions for biofilm production. Finally,basedon optical density (OD) bacterial films can be detected and classified into biofilm production or no biofilm production, this method is also validated by Stepanovicet al., (2000).

Recent study by Metzler (2016) revealed that commonly used crystal violent (CV) method can be used to quantify biofilm production by *S. aureus.*Thismethod is preferred over other methods due to its simplicity, reliability and fast nature. Interestingly, the assay also allows categorizing the isolate into high, moderate and non-biofilm producers. The action of the CV includenon-specific staining of all the biomass, both living and dead, as well as the matrix consisting of extracellular polymeric substances. This stain makes the assay useful to assess the overall biofilm response of an isolate .

3. MATERIALS AND METHODS

The study was conducted at the Microbiology Laboratory Faculty of Medicine,Near East University (NEU) Hospital, Nicosia, Cyprus from November 2017 to July 2018.

3.1. Equipment

Autoclave, bunsen burner, wire loop, conical flask, storage refrigerator, microtiter plate, weighing balance (PF-6001, Fisher brand), eppendorf tubes (microcentrifuge, 2.0ml), petri dishes, microscope, test tubes, glass slides, and incubator.

3.2. Bacterial isolates and growth media

A total number of the strains were 38 and were subjected to the standard microbiological method to isolate *Staphylococcal aureus* as indicated in Table 1. All specimens were identified at Microbiology Laboratory of Near East University Hospital.

- For the isolation of *S. aureus*, blood agar was prepared according to the manufacturesspecification. Blood agar is a type of growth medium which contains*trypticase soya agar enriched with 5%* human blood that encourages the growth of bacteria. The composition include the following,

- Papaic digest of soy meal
- Sodium chloride
- Agar
- Distilled water

3.3 Procedur for the preparation of blood agar

Forty gram of nutrient agar was added to 950ml of distilled water in a flask, and the composition was thoroughly mixed. Then, the composition was slightly heated and boiled for 1minute. The mixture was then autoclaved for 15minute at 15psi pressure at 121°Cthen it was allowed be cooled to 45-50°C. Aseptically, 50mL of sterile blood was added and mixed thoroughly, and poured into petri dishes for subsequent use.

3.4. Blood agar and determination of the haemolysis of the isolates

Production of extracellular enzymes by some bacterial species may cause breakdown of red blood cells (RBC) in the blood agar. The principle of diffusion is employed by haemolysin (exotoxin) outwardly from the colony (or colonies) resulting to complete or partial destruction of the RBC in the medium and complete denaturation of haemoglobin within the cells to colourless products. There are 4 types of haemolysis produced in blood agar by Staphylococci.These include; Alpha, haemolysis, Beta haemolysis, gamma haemolysis and alpha prime or wide zone alpha haemolysis. Haemolysis is best seen by examining colonies grown under anaerobic conditions or inspecting sub-surface colonies. These gives further classification of the isolate and differentiate from members of *Streptococcus species* and *Staphylococcus species*. The isolates were identified using cultural method, gram staining and biochemical tests to identify the specific isolate. In the detection of biofilm producing species, *Escherichia coli, Proteus species* and *Pseudomonas species*were used as a control because they are considered as non-biofilm producers. These isolates were used to check if they produce black colonies as an indication of biofilms production.

3.5 Congo Red Agar (CRA) preparation

As directed by the manufactures specifications, 10gram of nutrient agar was measured using weighing balance, 0.4gram of Congo Red and 7.5gram ofNaCl were added into conical flask (Fig. 3.1). Two separate medium were prepared using

different sugars at the same concentrations and named agar 1 and agar 2 to contain glucose and sucrose respectively.

Fig. 3.1 CR medium

Then 500ml of distil water was added. Glucose (10gram) was added and autoclave at 12°Cfor 15 minutes. CRA was inoculated with the test organism and incubated for 24 hours at 37°CFor the indication of biofilm producing organism, black colonies were expected (Agar 1).

For the determination of effect of different sugars, same media preparation was used with different type of sugar in Agar 2 (Sucrose) at the same concentrations. The media preparation is described in Table 3.1. To the different media (Agar 1 and

Agar 2), after autoclaving, 80ml of glucose syrup was added to the agar 1 preparations and 80ml of sucrose was similarly added to agar 2.

Table 3.1. Different media preparation constituents

Agar 1	Agar 2
500ml of distilled water with the following:	500ml of distilled water with:
(1) 0.4 gram of Congo red, 10grams of nutrient agar then 7.5gram of NaCl were added to flask	(1) 0.4 gram of Congo red, 10grams ofnutrient agar and 7.5 gram of NaCl
(2) To the 500ml of distilled water, 10gram of glucose was added and autoclaved for one hour	(2)To the 500ml of distilled water, 10gram of sucrose is added and autoclaved for one hour
(3) After cooling 80mL of glucose was taken from flask, and added into flask 1, shake and poured into petri dishes	(3)Similarly, 80mL of sucrose was taken from flask 2, shake and poured to petri dishes.

Nutrient broth was prepared as follows; 1.6gram of nutrient broth was added to 200 ml of distilled water and heated for 10 minutes and 5ml of the broth dispensed into test tubes. Then incubated for one hour at 37°C.

To each test tubes containing broths, 100 ml of sucrose were added, and bacteria was inoculated and mixed, then incubated for one day at 37°C. This is applied to the Staphylococcus colonies and the control isolates. Similar procedure was done using glucose and to all bacterial isolates.

As control to the experiment, three bacterial strains were used, which include:

E. coli, Proteus species and Pseudomonas species were incubated on the CRA plates to detect whether or not they produce black colonies. All the control species were incubated on both Agar1 and Agar 2. The incubation was done for 24 hours at 37°C.

3.6.Identification of biofilm using microscopy methods

Aloop full of test organisms was inoculated into 41 sets of broth, 38 test tubes for the sampledorganisms while 3 for the control. The test tubes were divided to contained two different sugars, into each set; 100µL of glucose and sucrose were added respectively. All sets were incubated for 24hours at 37°C(Fig.3.2).

Fig.3.2. Broth containing *Staphylococcus species* in test tubes

After incubation, 50µL of the test organisms from the test tubes were transferred into eppendorf tubes, then 20µL of indianink was added and 1ml of distilled water were adequately mixed. Certain portion of the mixture was transferred on slides and

viewed under microscope for possible formation of biofilm ring around the colonies(Fig.3.3).

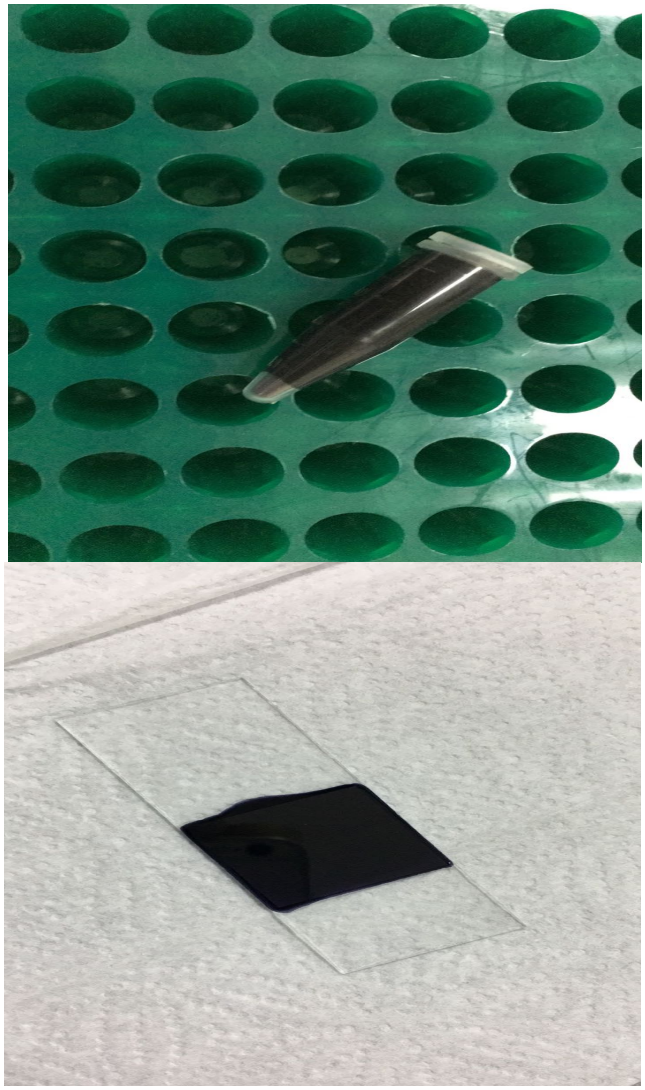

Fig.3.3. Mixture of indian ink and isolate of interest on a microscope

4. FINDINGS

This thesis was conducted atMicrobiology Laboratory, Near East University Hospital from July until August 2018.

A total of 38strains were collected from the patients attending NEU Hospital, and subjected to microbiological analysis to isolates *Staphylococcal species*, and are being classified as α,β haemolysis and non-haemolysin species, other classifications were based onmucoidity of the species(Table 4.1).

Table 4.1. The mucoidity and haemolysis characteristics of the isolates

Strain Number	Mucoid level	Haemolysistype	Source of the strain
231	+++	A	Aspiration
236	++	B	Sputum
242	+++	A	Blood
247	++	B	Wound
251	+++	A	Catheter
263	+	A	Blood
269	++	B	Wound
270	+	B	Wound
273	+	B	Nasal wash
275	+	B	Nasal Wash
276	++	B	Nasal Wash
290	++	B	Blood
291	++	A	Blood
299	+	A	Blood
312	++++	A	Catheter
329	+++	B	Wound
337	+++	A	Nasal wash
344	+	A	Throat wash
348	+++	B	Blood
351	+++	B	Blood
363	++	B	Wound
364	++	A	Wound
372	++++	A	Asparation
375	++	A	Wound

377	++++	B	Nasal wash
380	++++	B	Blood
416	+++	B	Wound
437	+	A	Nasal wash
448	+++	A	Nasal wash
456	+	A	Nasal wash
491	++++	A	Body fluid
492	++++	B	Sputum
494	++++	B	Blood
497	++++	A	Asparation
507	+	A	Catheter
509	+	A	Wound
537	+++	A	Nasal wash
550	+++	A	Nasal wash

Abbreviations:Key: ++++ complete mucoid, +++ strong mucoid, ++ good mucoid, + weak mucoid

4.1. CongoRed Agar (CRA) preparation results

The result of CRA supplemented with glucose and sucrose (2.5 grams each) after incubation for 24hours at 37°C is presented below (Fig. 4.1).

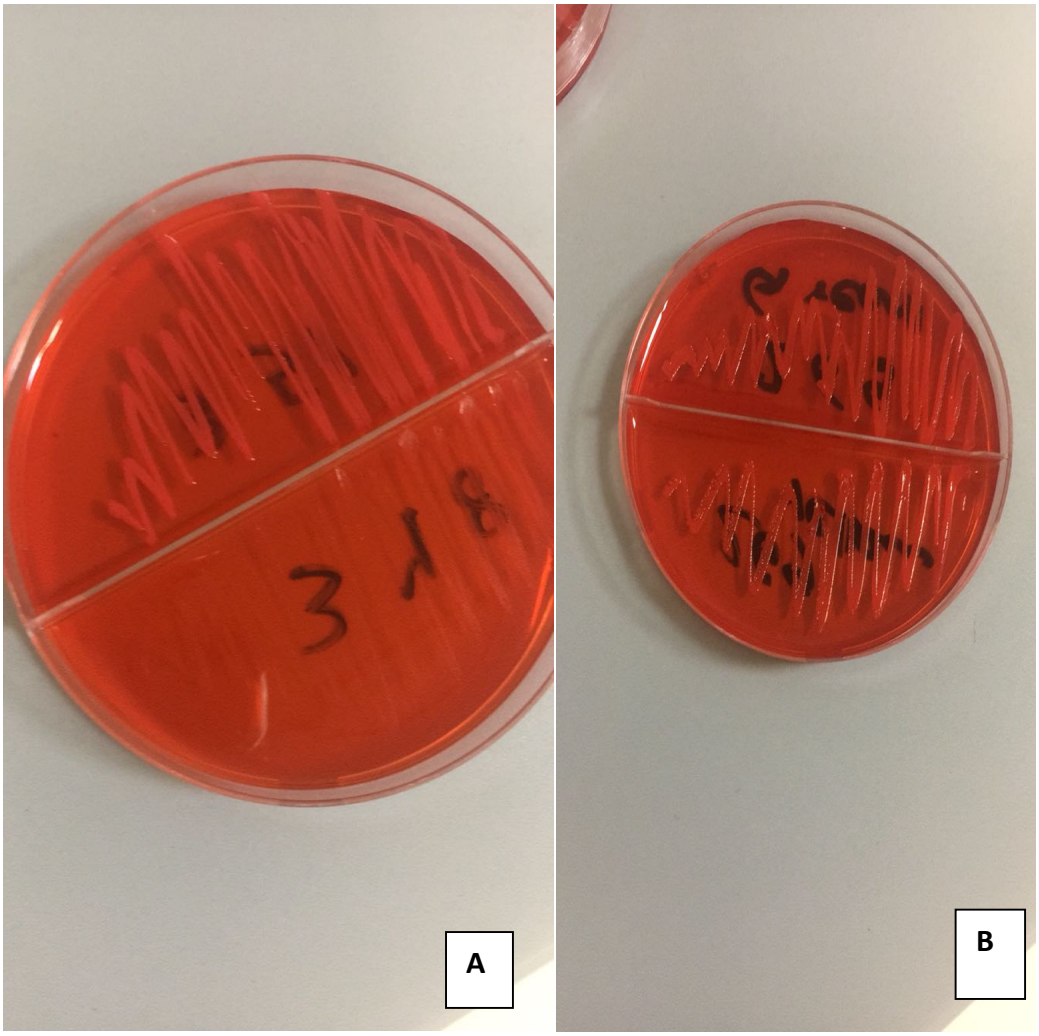

Fig. 4.1. CRA inoculated with the isolate after 24 hours incubation at 37°C℃

Then, the incubation time was added to 48hours on CRA at 37°C and the result is presented below(Fig. 4.2)

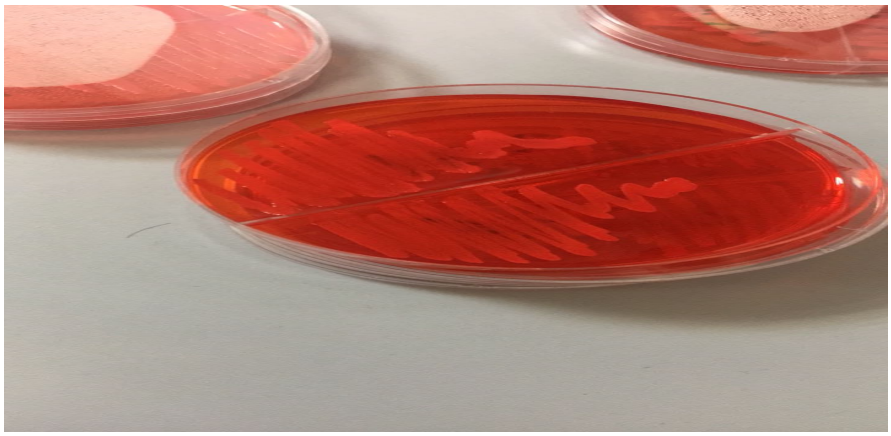

Fig. 4.2. Isolates on CRA after 48hours at 37°C .

For the determination of biofilm on CRA, two sugars were added to determine which was the best enhancerfor biofilm formation and the results are presented below(Fig. 4.3).

When CRA and the sugars were autoclaved, both the media and the colonies appeared black, these results were observed with both agar type 1 and agar type 2.

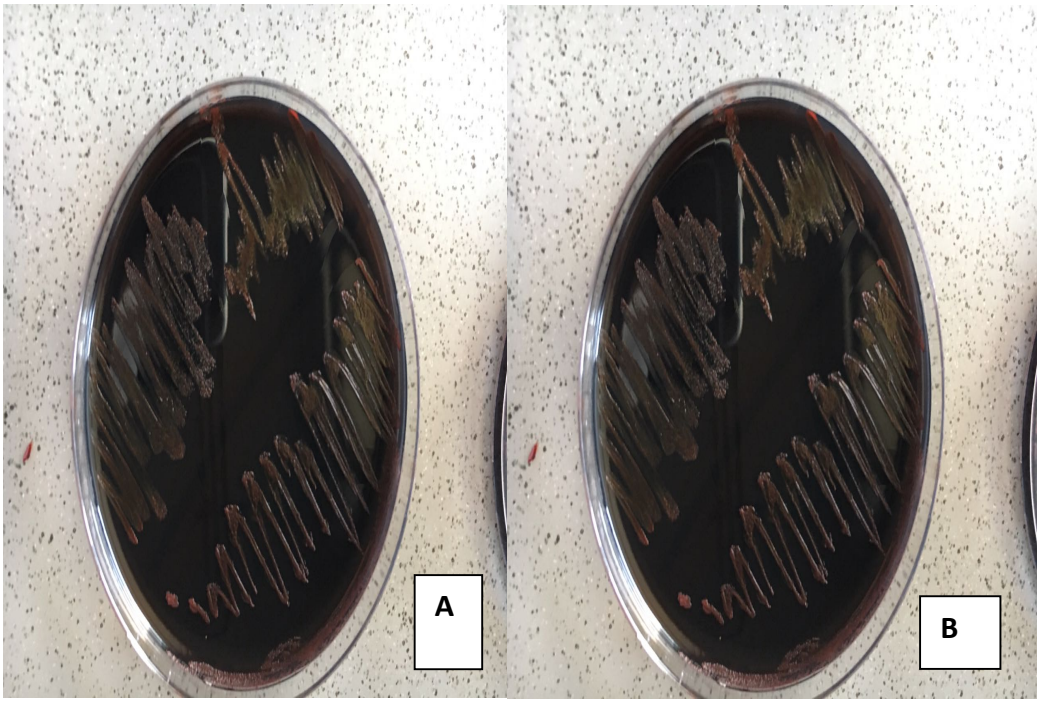

Fig. 4.3. Both CRA and the isolate turned black after 24 hours at 37°C(agar 1)**(**A-B)

A-CRA with glucosewhereautoclavedturned black color.

B- CRA with glucose where autoclaved turned black color.

Result obtained were similar on agar 1 and 2(Fig. 4.4). In both conditions (Fig 4.3 and Fig 4.4) direct addition of sugar to the agar medium during autoclaving resulted to burning of the sugar, hence it was difficult to differentiate between the medium and the black colony formation.

Fig. 4.4. Staphylococcuson CRA containing sucrose (agar 2)(A-B)

A- CRA with sucrose where autoclaved turned black color.

B- CRA with sucrose where autoclaved turned black color.

After the initial observation of burning of sugars when added to media during autoclaving, modification was made. The addition of the different sugars (80mL of

both glucose and sucrose) to the CRA was added after autoclaving the medium. The result of the modified composition is presented below for each sugar.

All of 38 of *S. aureus* isolate form biofilm(Fig. 4.5.1)(Fig. 4.5.2)(Fig. 4.5.3)

(Fig. 4.6.1)(Fig. 4.6.2)(Fig. 4.6.3).

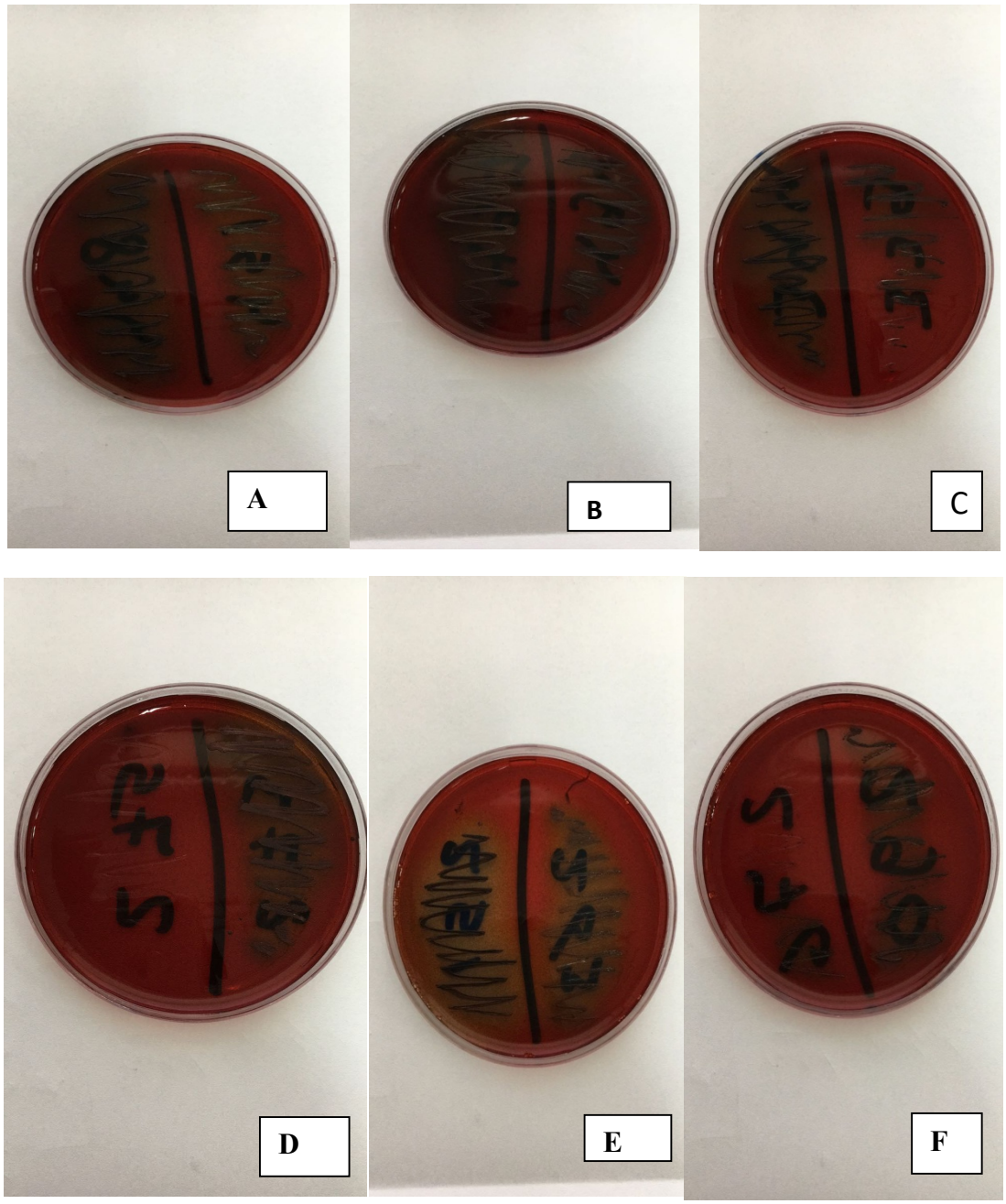

Fig. 4.5.1. Modified composition of nutrient agar (10gram)with 80mL of glucose (A-F).

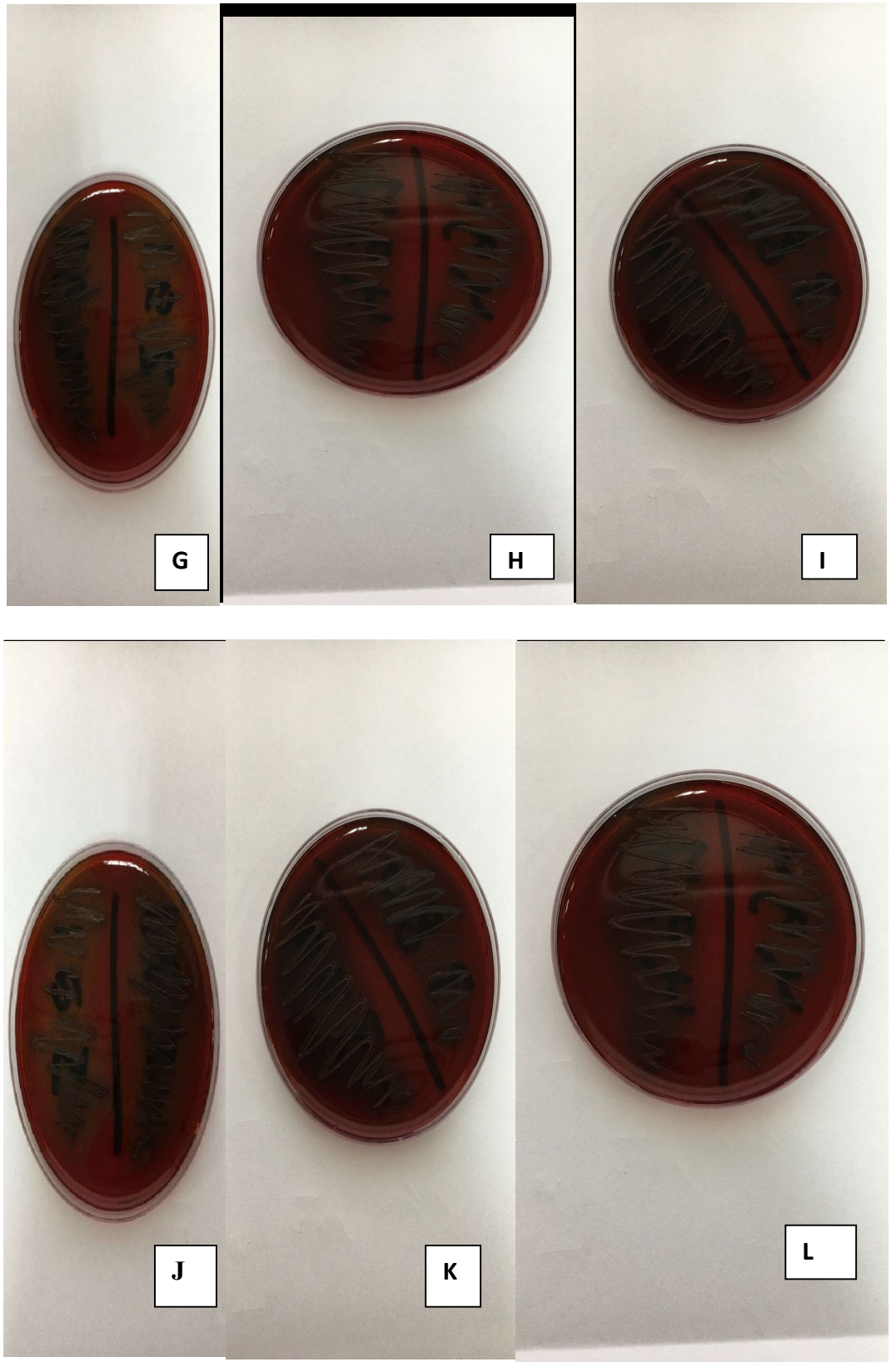

Fig. 4.5.2 Modified composition of nutrient agar (10gram) with 80mL of glucose (G-L).

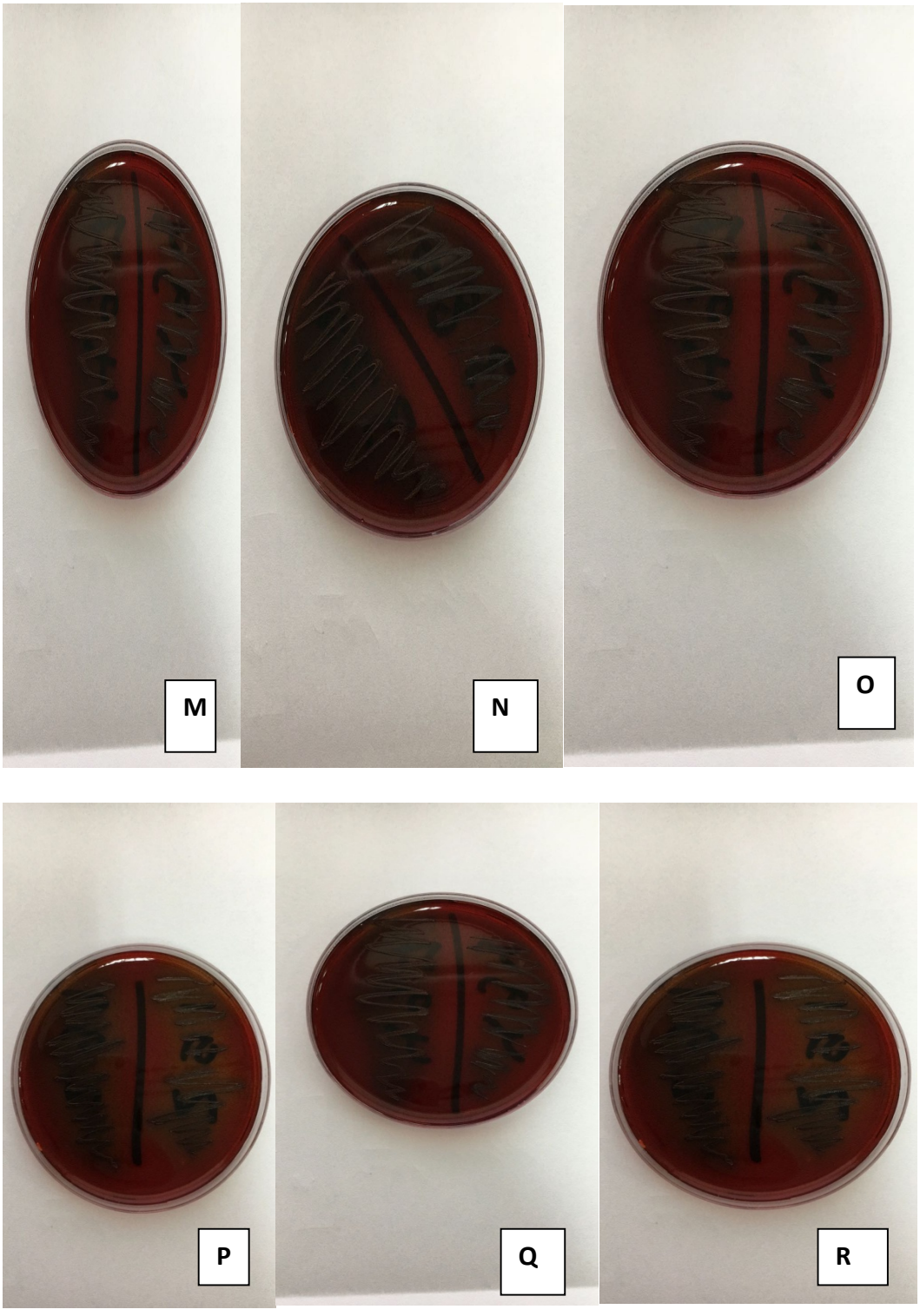

Fig. 4.5.3 Modified composition of nutrient agar (10gram) with 80mL of glucose (M-R).

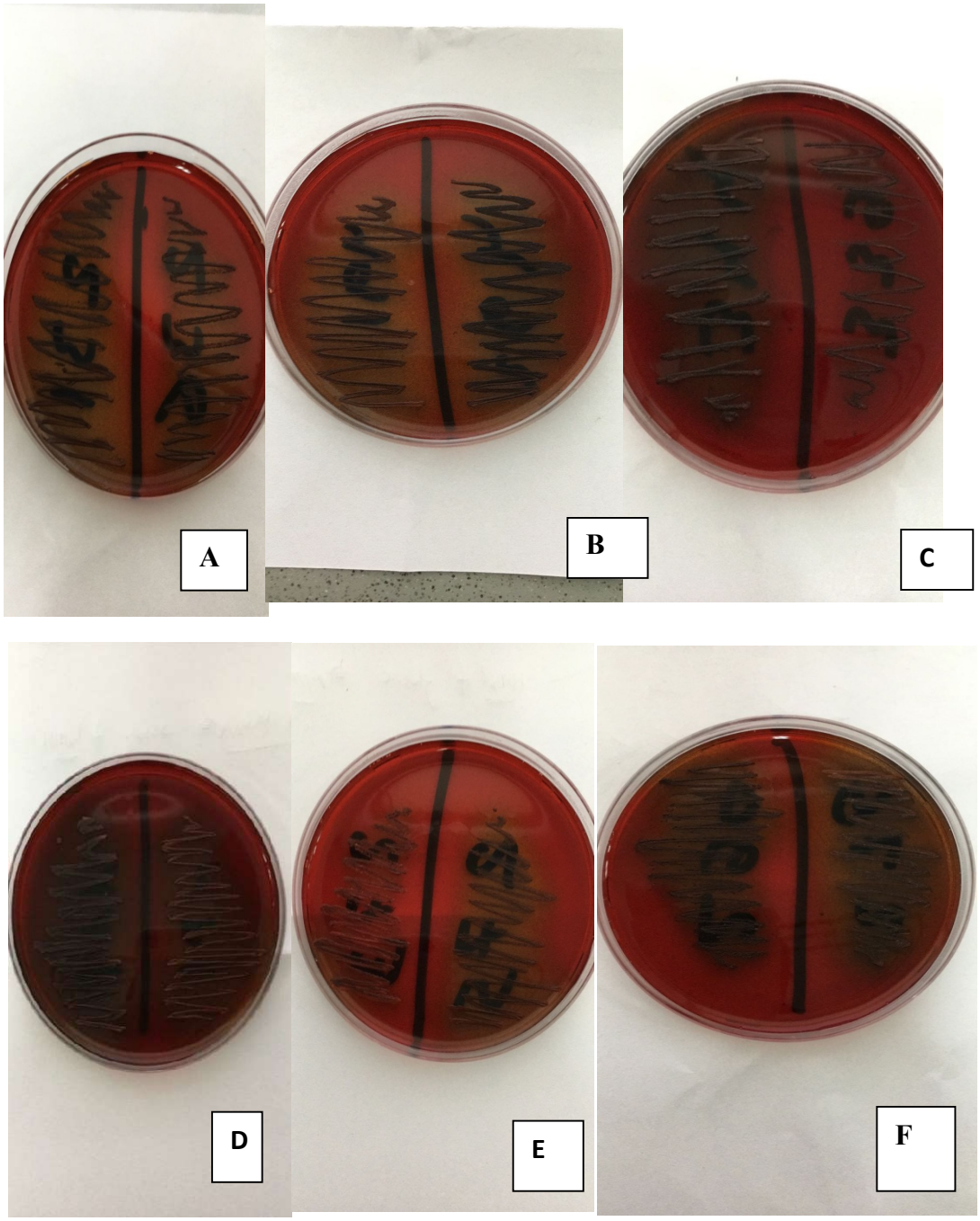

Fig. 4.6.1 Modified composition of nutrient agar (10gram) with 80mL of sucrose (A-F).

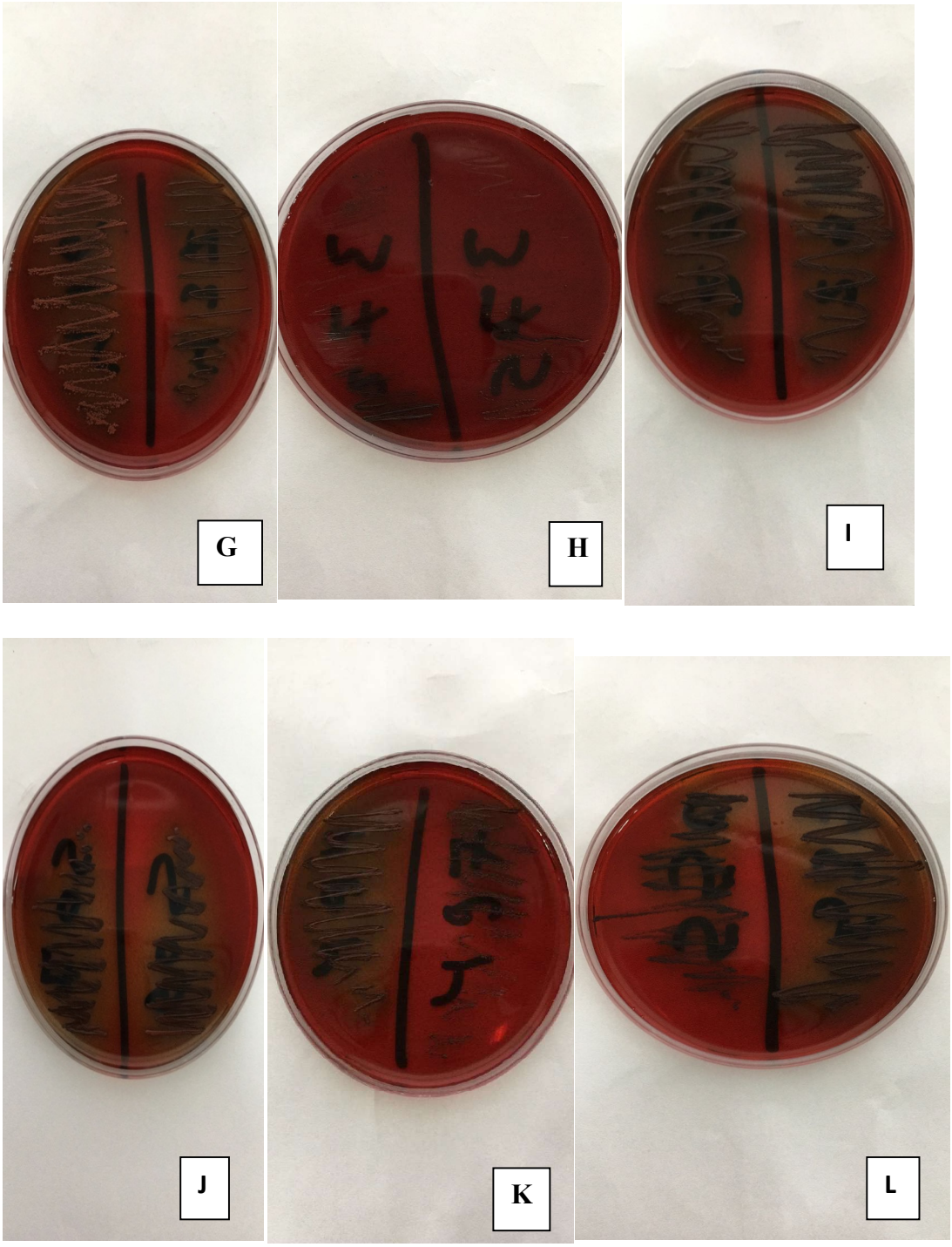

Fig. 4.6.2 Modified composition of nutrient agar (10gram) with 80mL of sucrose(G-L).

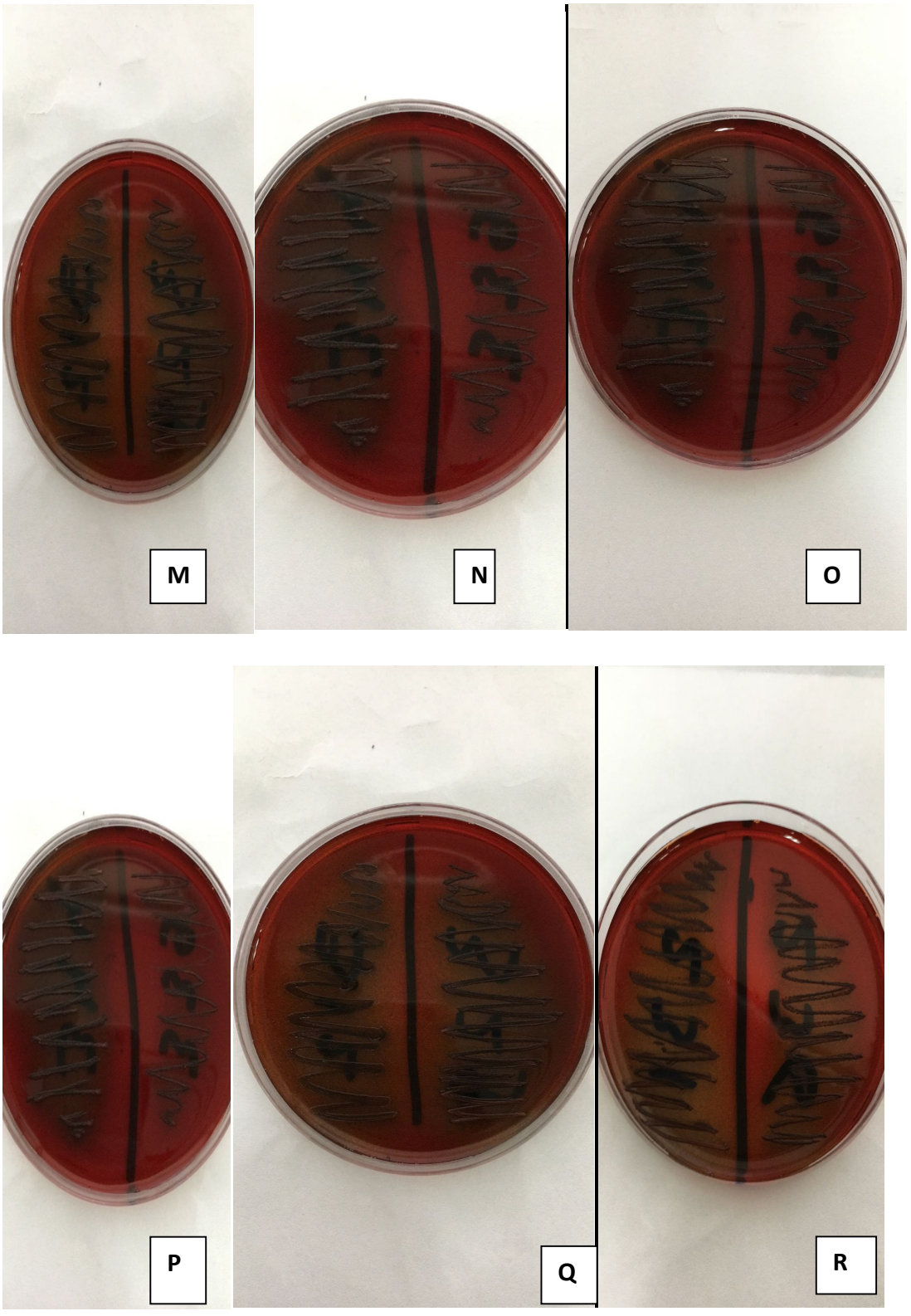

Fig. 4.6.3. Modified composition of nutrient agar (10gram) with 80mL of sucrose**(**M-R).

The control strains used in this study are considered to be non-biofilm producers and the results are presented below(Fig 4.7) (Fig. 4.8).

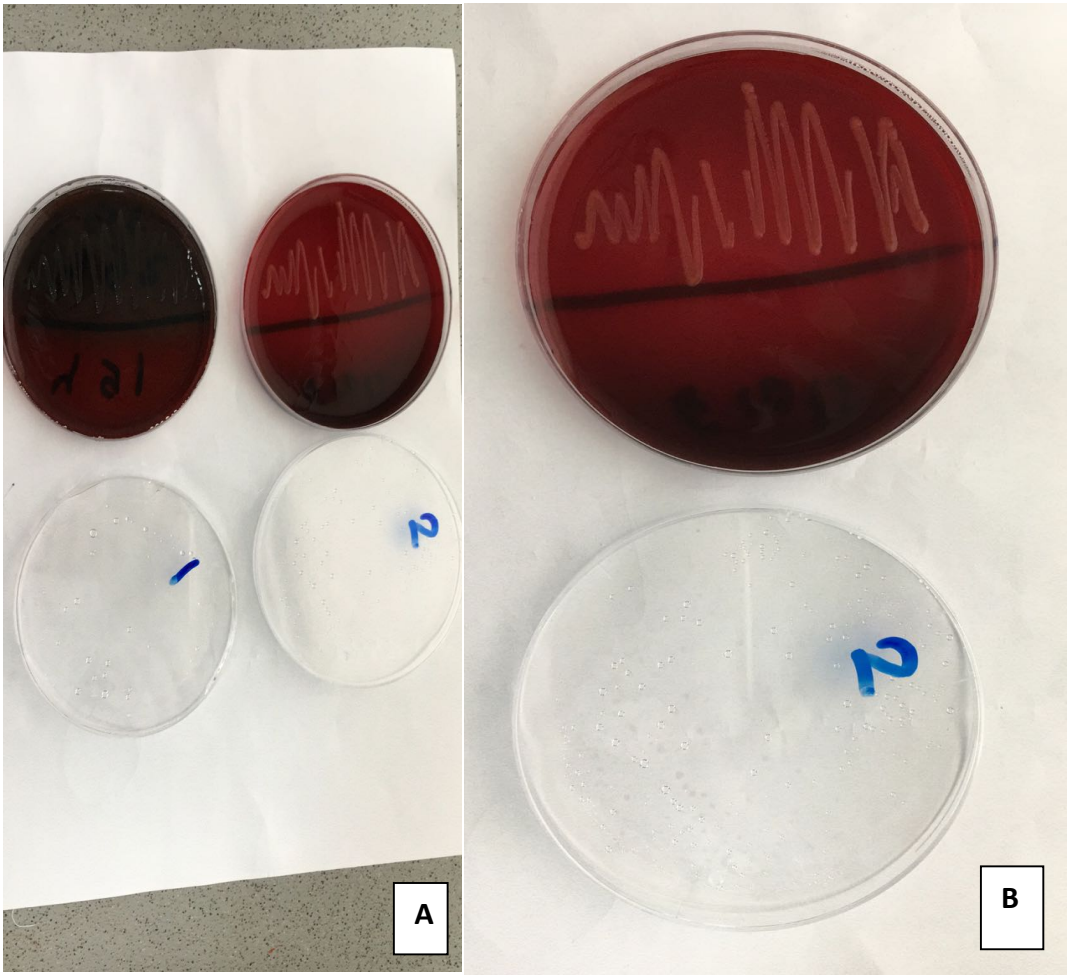

Fig 4.7.*E. coli* used as a negative control; no biofilm formation detected(A-B).

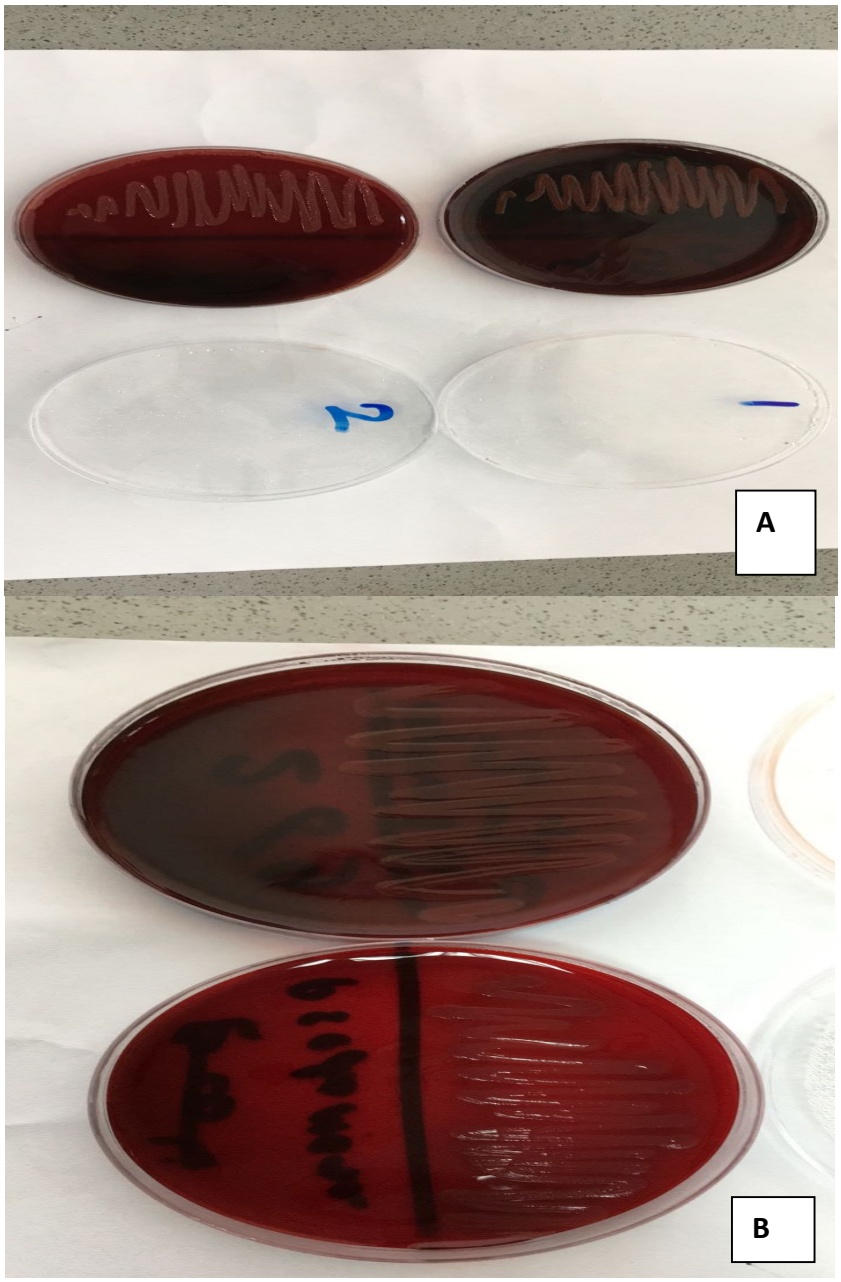

Fig. 4.8. (A)*Proteus species*used as a negative control; no biofilm formation detected.

(B)*Pseudomonas species* used as a negativecontrol; no biofilm formation detected.

4.2. Test tube results

Determination of biofilm formation on broth containing *Staphylococcusspecies*in a test tubes and the result is presented below,all of 38 of *S. aureus* isolate form biofilm(Fig.4.9)(Fig.4.10).

Fig.4.9. Broth containing Staphylococcus species

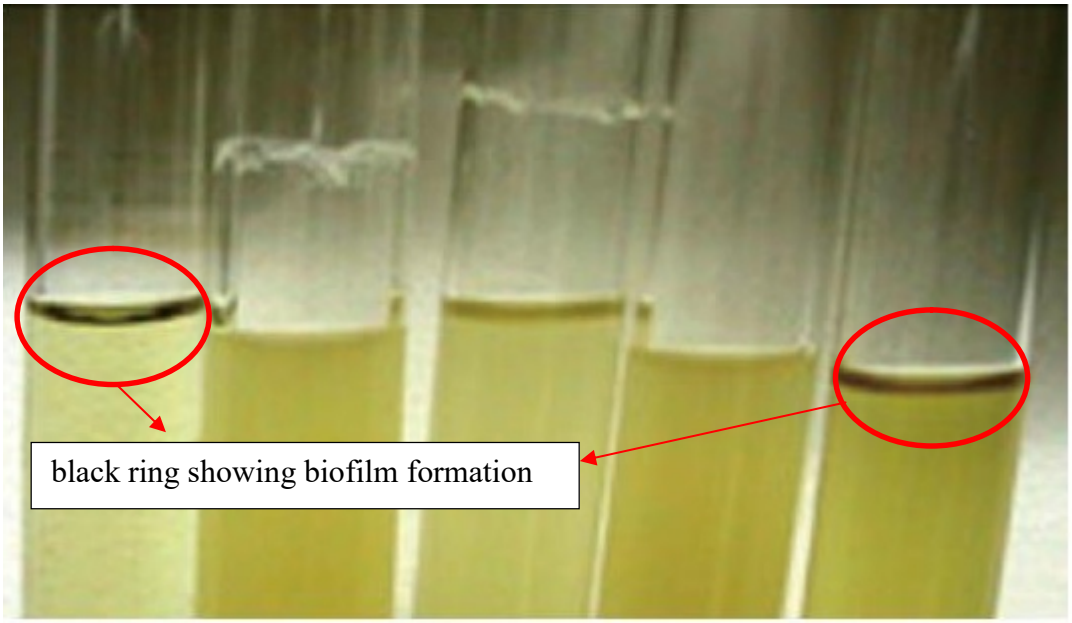

Fig. 4.10. Staphylococcal biofilm in a test tube, indicating ring form biofilms. Inoculation of the isolates into broths and monitoring the turbidity that indicated biofilms.

4.3. Microscopy method results

The results of staining techniques using indian ink on the species and viewed under microscope. The result is presented in fig 4.11 (A-D) all of 38 of *S. aureus* isolate form biofilm.

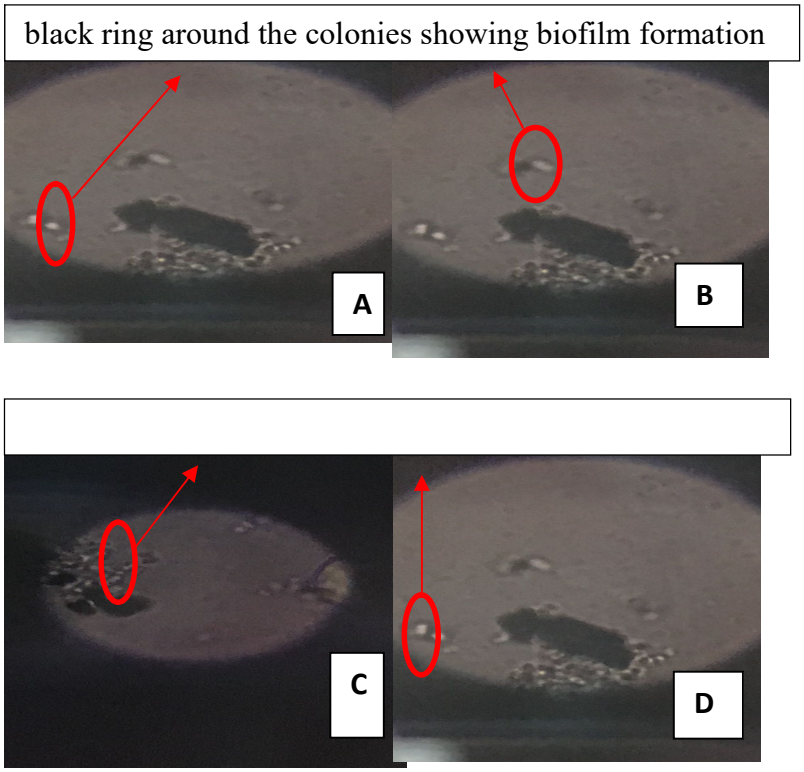

Fig. 4.11. Biofilm surrounding the colonies under microscope (A-D).

4.4. The Comparison between CRA and Microscopy methods

The two methods broadly used for the phenotypic identification of biofilm producing strains are the Microscopy methods and the Congo red agar (CRA) method(Christensen et al ., 2008). The Microscopy methods was developed to replace the test tube method, which was the first method used for microscopic estimation of bacterial biofilm on the surface of plastic slide. The Microscopy methods uses to measure the optical density (O.D) of stained bacterial biofilms and produces quantitative results.

CRA is considered preferred method for being more practical technique and require less time to detect biofilm in the agar, directly after autoclave the media, and clearer

than Microscopy method, also use less equipment. CRA was more of a reliant method,it was based on a subjective of chromatic evaluation (Freeman et al., 1989).

The advantage and disadvantage of CRA and Microscopy method are present in the table (5.1).

Table 5.1: The Comparison ofCRA and Microscopy methods

	Microscopy method	(CRA)
Quantitative detection	Quantitative	Not Quantitative
Time take to detect biofilm	Long time	Less time
The dyes use to detect biofilm	Indian ink	Congo Red
Uses for practical	Less practical	More practical

5. DISCUSSION AND RESULTS

Biofilm can be defined as a complex microbial derived sessile community of cells that are firmly held cling to surfaces and are embedded in a matrix of extracellular polymeric substance. The phenotype of a biofilm is dependent on the mode of growth, expression of specific genes and the production of biomolecules such as proteins. The biofilm can be composed of single specie or mixture of species; the environmental conditions that favour the establishment of the colonies. As stated, earlier biofilm can originate from a single cell, differential environmental conditions such as oxygen gradient and nutrients in the community can potential the development of distinct subpopulation of heterogenous gene expression. This phenomenon was demonstrated by the work of Archer et al., (2011) in a which model of Staphylococci invitro was made and four distinct metabolic states were identified which include cells glowing in aerobic condition, fermentative state, dormant state (including persisters) and dead cells.

Biofilms cause substantial problems in many environments and also contribute to the development of diseases and is known to cause problems during the treatment

of infections. Infections caused by biofilm-causing organisms are chronic in nature and mostly occur in hospitals. *S. aureus* is a major causeof biofilm-associated infections. Research by Periasamyet al.,(2011) identified *S. aureus* to have unique ability to attach to indwelling medical devices by either direct interactions with the device's polymer surface or by clinging to human matrix proteins after those proteins have covered the device. *S. aureus* is able to adhere firmly to surfaces to form a biofilm consequently responsible for many morbidity and infection (Zmantar et al., 2010). Most studies reported that bacteria in biofilms have the ability to resist action of physical stress and the washing action of saliva, and the organisms within biofilm can withstand nutrient depletion, pH fluctuation, oxygen radicals, disinfectant and antibiotics (Jefferson, 2004). There isrise in the medical device-relatedbiofilm infections which is very important in clinical settings because it can provide an extracellular barrier to antimicrobial agents or host immune system (Hsu et al., 2015).

In this study, two methods of detection of biofilm production by *S. aureus* were employed. Standard microbiological method was used to isolate *S. aureus* from the strains for the isolation of the test organism. A total of 38 S. *aureus* isolates were incorporated into this study, together with control strains *E. coli, Pseudomonas species* and *Proteus species*. The current study faced issue of not producing the required colonies on the first set of the experiment, in which the cultural method did not yield positive black colonies, an indication that biofilm was formed, this was largely believe to be due to lack of adequate sugar (polysaccharide) in the media and the test organism could not produce enough, the chief component of biofilm; extracellular matrix. Periasamyet al., (2011) indicated that ECM contribute to intracellular aggregation which helps in proliferation, furthermore, the authors revealed that in Staphylococci*,* the matrix consist of many secreted polymers such as exopolysaccharide, teichoic acid and specific proteins as well as DNA from lysed cells.

Several studies have investigated biofilm production by S. species using different methods (Cassetet al., 2007; Croeset al., 2009; Zmantaret al., 2010; Walker and Horswill, 2012;Metzler, A., 2016; Shukla and Rao, 2017; Singh et al.,2017;

Torlaket al.,2017). In this study, it was found that all the methods used in the work, were able to detect biofilms formation amongst the isolated strains. In the current study biofilms production/ESP production by the 38*S. aureus* strains were assessed by the production of either biofilm black colonies on (CRA) the formation of a turbid ring in a tube or indian ink surrounded the cells, indicating presence of EPS and can be viewed under microscope. This study confirmed the previous studies by Gundogan et al., (2006), Vasudevan et al.,(2003) and Rohde et al., (2007).These studies indicated that significant numbers of *S. aureus* are slime producers. Interestingly, the control used *E. coli*, *Pseudomonasspp*.and *Proteusspp*.were not biofilms producers (Fig. 4.8 (A-B); 4.9 (A-B)) as demonstrated that Rasamiravaka et al., (2015) and Mulcahy et al., (2014) for *Pseudomonas*, Kwiecinska-Piróg et al., (2014) and Jacobsen and Shirtiff (2011). This strange observation could be (due to the difference of the method employed in this study with the previous studies. In many biofilm-forming bacteria, progression of the planktonic state into sessile state is influence by environmental factors, were not studied in the current study.

The striking observation made in this study is the indication of the role of polysaccharides in the formation of biofilm. In the current study, it was found that the sugar (glucose - sucrose)enhanced the formation of biofilm .This was evident that the first set of CRA with no sugar supplementation did not yield biofilm colonies. This observation agreed with previous studies by Waldrop et al., (2014), Khangholi and Jamalli (2016). In these studysugar, glucose where added to detect biofilm formation by *S. epidermidis* and *S. aureus* in Lennox broth, the concentrations were given in an increasing manner from 0 to 320 mg/dL in 20 mg/dL intervals. Biofilm was grown for 24 hours for *S. epidermidis* and 48 hours for *S. aureus.* Furthermore, Khangholi and Jamalli (2016) demonstrated that biofilm mass was increased at higher glucose concentration for both species with a threshold response at 0 to 20 and 160 to 200 mg/dL for *S. epidermidis* and 200 to 240 mg/dL for *S. aureus*, similar to observed in this study when high concentration of glucose and sucrose were used, the black colonies appeared more readily.

This was in agreement with the method of Kwasny and Opperman (2010), in which a glucose-free medium suitable for bacterial growth and added known

amounts of glucose to produce specific glucose concentrations. Bacteria were grown overnight in glucose-free Lennox broth and then diluted 100-fold in Lennox broth containing one of 17 glucose concentrations from 0 to 320 mg/dL in 20-mg/dLintervals.Period of incubation plays role in the formation of biofilms as demonstrated in this study, where incubation for 48 hours produced enough biofilm compared to 24hours incubation with little biofilm formation. This also in tandem with the work of Kwasny and Opperman (2010) where two Staphylococcal species were incubated at different period; *S. epidermidis* was incubated for 24 hours and *S. aureus* for 48 hours; period of incubation play role as indicated that the adequate period was necessary to produce enough biofilm mass for assay. There was no evident observation in the difference of the role of glucose and sucrose in the current study, what is obvious was their role in enhancing biofilm formation, also the addition of the sugars should be after cooling of the medium which would not affect the media preparation. Thus, the presence of glucose and sucrose lead to the development of a stronger biofilm colonies. Pereira et al.,(2015) extensively studied the role of glucose in the proliferation of biofilm matrix in the presence of high sugarconcentrations, which could be a reason for the fact that sugars play important role in bacterial growth and metabolisms. In another study, different sugar, galactose was showed to facilitate formation of *B. subtilis*, a gram positive bacteria like *S. aureus* (Yunronget al., 2012). Consistently with this study, the authors revealed that sugar required for the biosynthesis of EPS as a nucleotide sugar substrate and thus for matrix production. Hence, it is obvious that sugar metabolism plays a central role in biofilm formation by bacteria.

In the tube methods, all the isolates turned out to be biofilm producing organism. Similarly, this method correlated well with the culture method on CRA for detection of biofilms. Though, it is difficult to differentiate the strength of the biofilm production. This work agreed with the Hassan et al.,(2011). Furthermore, Hassan et al., (2011) suggested that tube method cannot be employed as a general screening test to detect biofilm producing bacteria. Interestingly, another study by Ruzicka et al., (2004) noted that tube method detected more biofilm in *S. epidermidis* than culture method. In the work,the authors found out of 147 isolates of *S. epidermidis*, Tube method detected biofilm formation in 79 (53.7%) and CRA detected in 64

(43.5%) isolates. They showed that TM is better for biofilm detection than CRA. In the same trend, in another study by Baqai et al., (2008), the tube method showed high biofilm formation than CRA, and concluded that CRA method showed very little correlation with the other methods and parameters of sensitivity, specificity and accuracy.

This study also employed use of microscope to detect biofilm formation by *S. aureus.* Several imaging methods have been reported to have detected biofilm formation and cell viability as demonstrated in previous studies (Joana et al., 2015). However, light microscopy remains a useful base-line technique to provide a visual identification of biofilm formation. In the current study, Indian ink was used but other authors also suggested various dyes such as periodic acid-Schiff (PAS), Haematoxylin and eosin (H&E), and brown and brenngram staining have been recently reported to be more practical, cheaper and reliable methods for detection of bacterial biofilms in different infection foci (Akiyama et al., 2003; Bulutet al., 2014; Davis et al., 2008; Hochstimet al., 2010; Oates et al., 2014; Tothet al., 2011; Wintheret al., 2009; Zhang et al., 2009). The detection of biofilm by these practical and cost-effective staining methods have been described as quantitative detection of biofilm biomass.

Findings in this study concluded that *S. aureus* form a biofilm and sugars play an important role in enhancing the formation of biofilm. This finding is clinically significant because biofilm production is associated with pathogenicity of organisms causing device related implant infections. Among the two methods used in this study, it was demonstrated that all detect presence of biofilm formation by the isolates but CRA was more of a reliant method.

Since this study only demonstrated the presence of biofilm in the isolates quantitatively, it is recommended that further study should conducted using the molecular mechanisms. There is a need for more information on the mechanism of biofilm formation at a molecular level and observe its association with other microbial processes such as virulence and antibiotics resistance.

The use of molecular methods such as the polymerase chain reaction (PCR), which amplifies the genes involved in biofilm production, will significantly help to supports the methods used in this study, also complements other methods used elsewhere. Basically, those methods are qualitative methods for instance tube adherence test and the (CRA) and quantitative methods for example the tissue culture plate (TCP) assay, which are phenotypic techniques. The recommendation to further use molecular techniques of biofilms detection will not only help in the characterization of the genes in the formation of biofilms but also help to determine if such genes are associated with other organism's activity such as resistance.

The present study also recommends that hospitals should use of molecular techniques to have more understanding of pathogenesis of clinical isolates and all the constituents of the samples, this will also serve as confirmation of the phenotypic methods.

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