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Effect of biologically and chemically synthesized silver nanoparticles on biofilm forming bacteria isolated from catheterized Iraqi patients.

A Thesis

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Certificate of Examining Committee

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(قَالُواْ سُبۡحَٰنَكَ لَا عِلۡمَ لَنَآ إِلَّا مَا عَلَّمۡتَنَآۖ
إِنَّكَ أَنتَ ٱلۡعَلِيمُ ٱلۡحَكِيمُ)٣٢

((صدق الله العلي العظيم))

سورة البقرة

الآية **(٣٢)**

Dedication

To

The Soul of my dear mother

My dearest husband

Who affection, love and encouragement make me able to get such success

My beloved kids

Rahaf, Mousa and Adam

whom I can't force myself to stop loving

Shaimaa

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Shaimaa

Summary:

Silver nanoparticles was considered a powerful antimicrobial agents recently especially after increasing incidence of diseases associated with biofilm and multi-drug resistant pathogens which necessary required to find a novel path to eradicate that's challenge. So the aim of present study was to synthesize silver nanoparticles by biological method using bacteria (*Enterobacter cloacae*) to eradicate biofilm forming bacteria as phenotypic and genotypic levels and comparison with chemical synthesis of nanoparticles.

The study was carried out at the period of February/ 2017 to January/ 2018. Urine samples 65 were collected from catheterized inpatients who admitted to Al Diwaniya Teaching, Al Hilla Teaching, Al Qasim and Al Hashimiya Hospitals. The results showed that 58 (89.2%) gave positive growth, 28 (43%) as biofilm producer after phenotypically biofilm detection assay with congo red and tissue culture plate methods and genetically using polymerase chain reaction (PCR) for detecting *iacA*, *smaI* and *esaL* genes encoding biofilm for three bacterial isolates (*Staphylococcus lentus*, *Serratia fonticola* and *Pantoea* sp.) were the results established that all tested bacteria had own biofilm genes. All biofilm bacterial isolates were diagnosed and identified by VITEK2 system.

The bacterial isolates with biofilm formation were *Proteus mirabilis* as main bacterial isolates at 5 isolates followed by *Klebsiella pneumoniae* and *Pantoea* sp. as second at 4 isolates then *Pseudomonas oryzihabitans*, *Serratia fonticola* at 3, *Enterobacter aeruginosa* and *E. coli* at 2 and *Enterobacter cloacae* and *Yersinia enterocolitica* at one isolate to each one while gram positive bacterial isolates represented by *Staphylococcus lentus* only at 3 isolates

The antibiotics susceptibility test was done toward selected biofilm forming bacterial isolates (*Staphylococcus lentus*, *Serratia fonticola* and *Pantoea* sp) by disc diffusion, minimum inhibitory concentration (MIC) and VITEK antibiotic sensitivity testing (AST) method. The results revealed that *Staphylococcus lentus*, *Serratia fonticola* and *Pantoea* sp. were resistant to most tested antibiotics except azithromycin and imipenem which were sensitive for them at 100% for *Staphylococcus lentus* and *Serratia fonticola*, *Pantoea* sp. respectively.

The biosynthesis of silver nanoparticles was done by using *Enterobacter cloacae* (cell free suspension) as bio- reductant agent while chemosynthesis using sodium borohydride as chemical reductant agent, in both methods, the silver nanoparticles were detected visually by changing in color from yellow to brown color. Biological and chemical silver nanoparticles were characterized by several techniques. The UV-visible spectrophotometric showed absorbance peak at 400 nm and 390nm to biological and chemical types respectively, Fourier Transformer Infrared analysis (FTIR) revealed that carboxylic groups and polyphenolic groups are coated on the surface of both silver nanoparticles producing stabilized nanoparticles, and by scanning electron microscope (SEM) and size analyzer showed that size of biological synthesis silver nanoparticles at 63 nm and chemical synthesis at 25 nm, also SEM showed the formation of cubical, mono-dispersed nanoparticles.

The antimicrobial effect of synthesized silver nanoparticles were evaluated by agar well diffusion and macrodilution method to determine MIC value. The results show that biological silver nanoparticles were more effective on biofilm forming bacteria (*Staphylococcus lentus*, *Serratia fonticola* and *Pantoea* sp.) than chemical synthesized one at $p \leq 0.05$. Furthermore the antimicrobial activity of combination between silver

nanoparticles in both types and antibiotics (azithromycin and imipenem) on growth of biofilm forming selected bacteria with control, approved that the combination display a synergistic effect which lead to enhance the antimicrobial activity of silver nanoparticles than its alone

Gene expression of biofilm encoding genes (*icaA, smaI and esaL*) were evaluated by Real- time quantitive polymerase chain reaction (RT- qPCR) before and after treatment with silver nanoparticles in both types and selected antibiotics (azithromycin and imipenem) and combination between them, the results revealed that biological silver nanoparticles alone or in combination with antibiotics were more effective on biofilm gene expression by down regulation than others treatments at $p \leq 0.05$.

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List of Abbreviations

Abbreviation	***Meaning***
^{0}C	Degree of Celsius
$AgNO_3$	silver nitrate
AHL	acyl-homoserin lactones
CAUTI	Catheter associated urinary tract infection
CFU	Colony forming unit
CLSI	Clinical and Laboratory Science Institute
C_T	Cycle threshold
CVS	Crystal Violet Stain
DLS	Dynamic light scattering
DW	Distal water
eDNA	Extracellular DNA
EPS	extracellular polymeric substance
FTIR	Fourier Transform Infrared Spectrometer
ica gene	Intercellular adhesion gene
M	Molarity
mg	Milligram
min	Minute
mL	Milliliter
mM	Mili Molarity
mm	Millimeter
N	Normality
$NaBH_4$	Sodium borohydride
nm	nanometer
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
OD	Optical density
PBPs	Penicillin binding proteins
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction

PIA	Polysaccharide intracellular adhesion
QS	Quarm sensing
RFU	Relative fluorescence units
ROS	Reactive oxygen species
rpm	Revolutions per minute
RpoB	RNA polymerase β subunit gene
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Scanning Electron Microscopy
SNPs	Silver nanoparticles
SPR	Surface Plasmon resonance
TCP	Tissue Culture Plate
TEM	Transmission electron microscopy
UTI	Urinary tract infections
UV	Ultra Violet
V	Volume
XRD	X-ray diffraction
µg	Microgram
µL	Micro liter

Appendices

Chapter One

Introduction and Review of Literatures

1. Introduction and review of literatures

1.1. Introduction:

Silver nanoparticles are used vastly today in biomedical, health care, food agriculture, industrial, electronics and environmental field. In medicine, the antimicrobial action of nanoparticles have the ability to destroy the wide spectrum of pathogens and multidrug resistance bacteria. Silver nanoparticles have used as a novel antimicrobial agents like antibacterial, antiviral, antifungal and anti-inflammatory agents. It act as antimicrobial agent for preventing biofilm attachment, penetration bacterial biofilm or delivering antimicrobial agents. Moreover silver nanoparticles can be synthesized by variety of different methods such as physical, chemical and biological. The last one is most favorable method because it safety, less toxicity and ecofriendly to environment while the chemical and physical methods are very costly, may contains a poisonous and dangerous materials (Chojniak *et al*., 2017).

Biofilm is an aggregate of microorganisms such as bacteria and attachment to biotic surface which protected by an extracellular polymer matrix composed of polysaccharides and extracellular DNA, it has widespread implications in the medical field (Neethirajan *et al*., 2014). According to reporting agency of the Center for Disease Control (CDC), the rate of bacterial infection associate with biofilm formation is estimated at 65% of all infections (Costerton, 2001) while the National Institutes of Health (NIH) estimate at 80% (National Institutes of Health, 2007).

Many microorganism associated with biofilm formation such as fungi and bacteria, the most common bacterial species as causative organisms of biofilm in urinary catheter are *Proteus mirabilis*, *Escherichia coli*,

Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, and Klebsiella pneumonia (Sousa *et al*., 2011). Biofilm formation after the reversible attachment, irreversible attachment, maturation and dispersion, bacterial cells gene expression during these stages alter and the bacterial cell become more "sticky" due to secret extracellular polymer matrix (EPS). It has many genes responsible and regulate of biofilm formation (attachment, colonization, adherent and quarm sensing)(Joo and Otto, 2012).

Due to increase use of a medical devices at high rate, biofilms represent a major source of contamination in medical and hospital settings, the rate of opportunistic infections lead to biofilm also Increased, such of them are pacemaker, Prosthetic valves, endotracheal tubes, intravenous and urinary catheter. Formation of biofilm on urinary catheter considered a major nosocomial problem. It is reported that urinary catheter responsible about up to 80% of nosocomial urinary tract infections (UTIs), and estimated that about 65% of human infections are related to the biofilm, the risk increase by 5–8% per day, rising rates of morbidity and mortality of patients (Costerton, 2007). Therefore, developing efficient strategies to combat bacterial biofilms is of the most important from medical and economic perspectives.

Antimicrobial materials used in the clinical setting today are beset by significant shortfalls, including weak antimicrobial activities, risk of microbial resistance, difficulty in monitoring and extending the antimicrobial functions, and difficulty in functioning in a dynamic environment. Thus, effective and long-term antibacterial and biofilm-preventing materials constitute an immediate need in medicine and dentistry (Beyth *et al*., 2015).

Eradication of biofilms often requires an antibiotic dose up to 1000 times higher than the lethal dose for planktonic bacteria (Gilbert *et al*., 2002). Bacterial biofilm tolerating antibiotics activity can also evade immune system by resisting phagocytosis, making biofilm more difficult to eradicate and causing challenging task for the physician and the microbiologists. This is important to develop a modulation treatment to increase antimicrobial penetration through biofilm layers. So it needs a novel path to eliminate biofilm formation.

The growing concern regarding multi-drug resistant bacterial strains and biofilm-associated infections calls for the development of additional bactericidal means. Consequently, attention has been especially devoted to new and emerging nanoparticle-based materials in the field of antimicrobial chemotherapy.

1.1.1.Aim of the study:

According to facts above, and in view to many local Iraqi studies focus on the antimicrobial effect of silver nanoparticles on biofilm forming pathogens by one method, no studies were done about the comparative between antimicrobial effect of chemical and biological synthesis method of silver nanoparticles and no study about effects on gene expression changing of biofilm pathogens, the present study was aimed to identify the role of biosynthesized silver nanoparticles in reduction the biofilm formation among multi- drug resistant pathogens according to phenotypic and genotypic levels. To achieve this aim the following objective were concluded:

1. Isolation and identification of biofilm forming bacteria from urine of catheterized patients.

2. Estimation the biofilm formation capacity among uropathogenic bacteria.
3. Identification of biofilm formation bacteria genetically.
4. Detection antibiotics susceptibility test to biofilm forming bacterial isolates using disc diffusion method and MIC by VITEK and macrodilution methods.
5. Synthesis of silver nanoparticles by chemical and biological methods.
6. Evaluation of antimicrobial activity of bio and chimosynthesis silver nanoparticles against biofilm forming bacteria and compare between them.
7. Evaluation the combination effect between biosynthesized and chemosynthesized silver nanoparticles and antibiotics.
8. Measurement the gene expression of gene encoding to biofilm for multi- drug resistant isolates before and after the treatment with the silver nanoparticles.

1.2. Review of literatures:

1.2.1. Biofilm formation:

Biofilms have been present back as 3.2-billion years ago (Rasmussen, 2000). The first microscopic visualization and discover biofilm bacteria in 1683 by A. Leeuwenhoek, when he scraped a layer of deposit from his teeth and examined under microscope (Costerton, 2007), while the first time originate the biofilm term was in 1977 by William Costerton when he observed the adherent of vast majority of bacterial community on the bottom's rocks than planktonic bacteria in an Alpine lake and described it as biofilm (Neethirajan *et al*., 2014). In reality, above of 99% of microorganisms in the ecosystem are exist as biofilm on a variety of surface (Vu *et al*., 2009).

Biofilm is a microorganism's cell community (bacteria, fungi, etc..), it is embedded in a matrix extracellular polymeric substance (EPS) composed of polysaccharides and extracellular DNA (eDNA) that attach and aggregate on several medical device such as (urinary catheter), when pathogen form biofilm, they resist the host immune defense and antibiotics action to cause severe illness and life threating infections (Christensen et al., 1985, Whitchurch et al., 2002). Bacteria can adhere to medical device surface by flagella protein, type IV pili, surface adhesin or by chemical and physical interaction and display alteration in phenotype, growth rates, metabolic activity, gene expression and product of protein(Archer *et al*., 2011, Lister and Horswill, 2014).

There are many potential reasons behind biofilms formation by bacteria. Some of them are defenses by evading the host immune system and protecting themselves from harmful conditions, colonization in region has richest in nutrients and sequester it and employment of

advantage cooperative system and live as community (Kokare *et al*., 2009).

1.2.2.Stages of biofilm formation:

To initiate the biofilm and converting the bacterial life style from planktonic to sessile form there are physical, chemical and biological interaction between the bacteria and biotic or abiotic surface, during these process, bacterial cells undergo a series of phenotypic and genetic changes.

Firstly, bacterial cells transported to selected surface by its appendages such as flagella, Pilli, fimbria then adsorption to surface by physical force include van der Waals forces associated with bacterial adhesion (Liu *et al*., 2004, Delcaru *et al*., 2016). This stage called reversible attachment.

Some of adherent bacterial cell remain attach to surface and arrested to be irreversible attachment. Bacterial appendages (flagella, pilli, fimbriae) and even EPS stimulate chemical reaction between bacterial cell and surface to unit bonds, which act as a bridge between bacteria and surface and that depend on degree of hydrophobicity and hydrophillicity of interaction surface.(Liu *et al*., 2004, Kokare *et al*., 2009, Joo and Otto, 2012).

Prolonger attachment of bacterial cell to surface predispose to communicate with each other's via signal small chemical molecules autoinducers called quarm sensing which in gram negative acyl-homoserin lactones (AHL) while in gram positive peptides (Chifiriuc *et al*., 2011) then bacterial cell start to aggregate to each other through cell to cell interaction then produce a matrix of extracellular polymeric substance (EPS) which composed of polysaccharides, lipid, protein and

extracellular DNA (eDNA) (Neethirajan *et al*., 2014, Delcaru *et al*., 2016). This matrix encapsulates bacteria to act as protective layer from undesired environmental conditions such as (antibiotics action, biocides, dryness, U.V radiation, stress factors and defense of host immune system) in addition to its role in reservoir of nutrient materials (Chifiriuc *et al*., 2011, Limban *et al*., 2013). Therefore it induce change adaption by separated bacterial cells from nutrients via slow metabolism rate and decrease oxygen and pH rate (Neethirajan *et al*., 2014).

The last stage of biofilm formation process is biofilm dispersal. There are many predispose factors inducing dispersion of biofilm, some of them are depleted of nutrient materials especially glucose deprived, EPS weakness, interfering with quarm sensing and attachment to produce protein's signal responsible on dispersion process (Huynh *et al*., 2012, Neethirajan *et al*., 2014). All that's stages are clear in (Fig. 1-1).

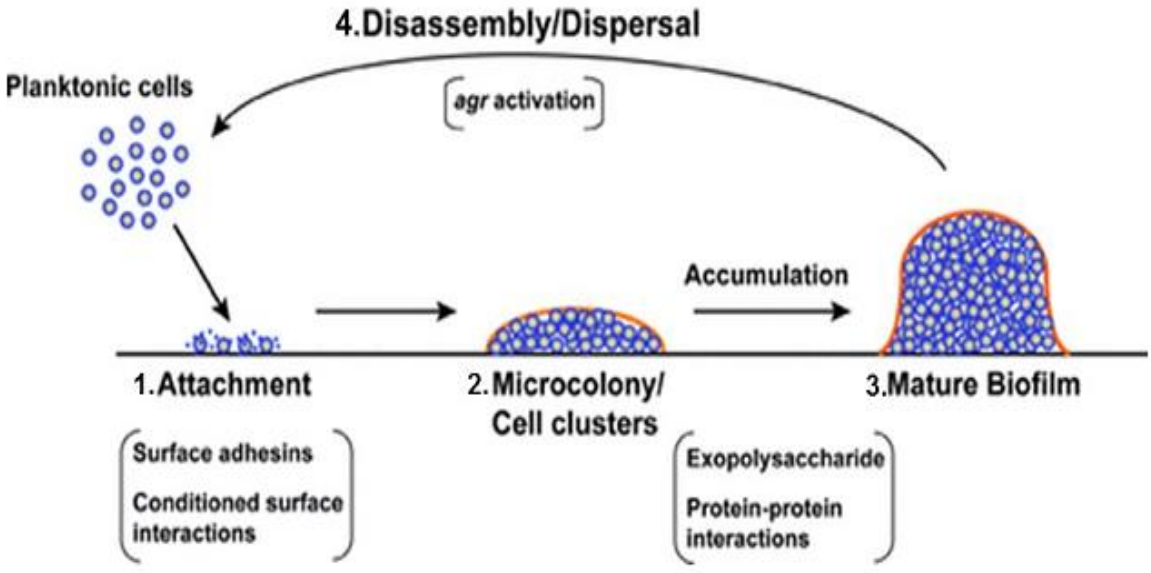


Figure (1-1): Biofilm formation stages (Wilkinson, 2016).

1.2.3.Quarm sensing (QS):

It is small chemical molecules called autoinducer, having main role in attachment and inducing bacterial cell to aggregate with each other to form biofilm (Li and Tian, 2012). Many genes are responsible to express the product of QS (acyl homoserine lactone (AHL)) and regulate its process such as (*smaI*, *esaL*, *lasR*, etc..) (Neethirajan *et al*., 2014, Ivanova, 2017). QS process different from bacterium to another according to its group (gram positive or negative), where gram positive bacteria use peptide or oligopeptide signals while gram negative bacteria utilize AHLs (Sifri, 2008, Galloway *et al*., 2012). AHLs accumulate at high concentration in threshold level in the surrounding environment then bind to transcriptional regulatory gene to induce target genes to express and activate biofilm formation (Choudhary and Schmidt-Dannert, 2010). QS sense the change in environment to give order to bacterial cell communicate with each other to form biofilm (Li and Tian, 2012, Cvitkovitch *et al*., 2003). Many factors influence the biofilm formation and induce QS, availability of nutrients, motility of bacteria, surface chemotaxises and surfactant present (Triveda and Gomathi, 2016).

1.2.4.Gene expression of biofilm formation:

When difference occurs in concentration of nutrients, oxygen and acceptor of electron, gene expression affected to be heterogeneous into biofilm. It also can be even different in cells neighboring to biofilm (Brady *et al*., 2007). In response to these environmental signals, sigma factor proteins and small non-coding RNAs (sRNAs) transmit regulatory signals to various genes to help the bacteria for adapt (Ghaz-Jahanian *et al*., 2013). The first step for many biofilms appears to be induction of a master regulatory gene that triggers the subsequent genetic changes

necessary for biofilm formation. Many of the gene expression changes are indeed highly conserved. Most relate to adhesion, and reflect an ability to adapt to the surface in question, that leads to be found in cells at different states (Neethirajan *et al*., 2014). The cells within biofilm grow at slow rate, fermentative and dormant state or dead while the cells outer biofilm grow at active metabolically and aerobically (Rani *et al*., 2007).

Each step of biofilm process regulate by genes families and each causative microorganisms have specific genes. A gene required for biofilm formation (causative genes) such as: numerous genes responsible of adhesion step such as : *icaADBC* (intercellular adhesin) act to synthesis polysaccharide intercellular adhesion (PIA) which assist *Staphylococcus* sp. and especially *Staphylococcus aureus* to adhesion solid surface and bind cell together to form biofilm (Jefferson, 2004, Oliveira and Cunha, 2008). There are many genes regulates the adhesion part of biofilm formation and quarm sensing regulation to synthesis the signal like *lasI* in *Pseudomonas* bacteria which have role to synthesis quarm sensing signal (Jefferson, 2004). While the genes which differentially regulate the biofilm process called (effective genes), most genes encoding biofilm affect by external environmental condition may alter express of some genes to form biofilm, that alteration maybe up or down to gene expression then lead to maturation of biofilm (Jefferson, 2004).

1.2.5.Microbial etiology of biofilm:

Biofilms may be formed by single or multiple species of microorganisms (Donlan, 2001). A vast variety of microorganisms form biofilm, fungi, yeast, gram positive and negative bacteria. Some of these microorganisms may form biofilm more easy than others and that related to it possess of extracellular organelles like Pilli, fimbriae or flagella

(Mortensen, 2014). Bacterial cell is cooperative to make communication between others via quarm sensing to regulate the water and nutrient supply and waste product removal (Hassan *et al*., 2011). That cooperation increase protection level so its explain the resistance antibiotics character of bacterial biofilm (Tarver, 2009).

1.2.5.1. Bacterial biofilm:

Most bacterial strain has ability to form biofilm some of them are:

1.2.5.1.1. Gram-negative bacteria:

Most Gram negative bacteria, prevalent as nosocomial infection related with UTI infection, associated with catheter (CAUTI) (Jacobsen et al., 2008, Johnson et al., 2006). Among the gram negative bacteria that are associated with biofilm infections is *Pseudomonas aeruginosa* which has established a more attention (Joo and Otto, 2012). *Proteus*, *Serratia*, *E. coli*, *Klebsiella* and other enterobacteriaceae are opportunistically infected urinary tract especially with catheterized patients (Holling, 2014), and have ability to form biofilm their which related to its pathogenicity to be important clinically (Pinna *et al*., 2008). Bacteria associated with catheter use are derived at large amount from patients gut microbiota (Kline and Lewis, 2016). Recently some bacterial strain enter the world biofilm such as: *Pantoea* sp.. The first report concerning this bacterium were published in 1972 and it was previously named *Enterobacter* and *Erwinia* and belong to enterobacteriaceae family (Bottone and Schneierson, 1972). It is opportunistic pathogen can cause human diseases by two ways; wound contaminated with plant materials or nosocomial infections (Dutkiewicz *et al*., 2016). Hospitalized patients especially those with immunocompromised infection may expose to contaminated equipment or fluids with these bacteria to happen infection

(Shubov *et al*., 2011, Boszczowski *et al*., 2012). The highest nosocomial infections cases with *Pantoea* sp. occur in USA hospital between 1970–1971in 152 septicemia cases out of 378 cases with mortality rate at 13.4% (Maki *et al*., 1976). Recently increase reported cases with *Pantoea* sp. in 2007 was reported as a causative agents to pediatric infections when isolate from various sites of the body involving urinary tract (Cruz *et al*., 2007). While in 2010, the cases decrease at low level to detect the good response to antibiotics treatment (Lee *et al*., 2010). But it return to appear in 2012 as nosocomial outbreak in hemodialysis patients and other cases associated with chronic renal failure (Kazancioglu *et al*., 2014).

1.2.5.1.2. Gram-positive bacteria:

Gram positive bacteria were less often biofilm formation than gram negative. Increased pathogenicity of these microorganisms is caused by the presence of many virulence factors, particularly the ability to form biofilm, the ability to co-aggregate, or the ability to withstand the effect of antibiotics. Strains producing extended-spectrum-b-lactamases (ESBL, *ampC*) and MRSA can be a particularly important problem (Hola and Ruzicka, 2011).

Among the gram-positive bacteria found in device associated biofilms are *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. *S. aureus* and *S. epidermidis* and other *Staphylococcus* sp. are the most frequent nosocomial infection causative agents on urinary catheter devices (Otto, 2008), and associated with biofilms which are regarded as most important causative agent associated with biofilm formation (Joo and Otto, 2012). The most critical pathogenicity factor in these bacteria is the colonization of abiotic or biotic surfaces by the formation of a three-dimensional biofilm. To form a biofilm,

staphylococci first attach either to host tissue or to the surface of a medical device, and then proliferate and accumulate into multilayered cell clusters, which are embedded in an amorphous extracellular material that mainly is composed of N-acetyl-glucosamine, cell wall teichoic acids, DNA, and host products (Mack *et al*., 1996).

1.2.6.Factors influencing rate and extent of biofilm formation:

At first step of biofilm formation, the bacteria must attach to exposed advice surface for long time to be irreversible. The rate of attachment depend upon three factors; the type and number of bacterial cells in the liquid which flow in advice, the rate of liquid flow and the physical and chemical characteristics of the surface (Donlan, 2001). Also the components of liquid may affect characterization of surface and therefore affect attachment rate (Garrett *et al*., 2008) When the bacterial cells attach irreversibly and extracellular matrix production, the growth rate is influenced by composition of nutrient, flow rate, environment temperature and concentration of antibiotics (Donlan, 2001).

1.2.7.Urinary catheter:

Urinary catheter is a tubular latex used in clinical purpose to transmit the urine from urinary bladder through urethra to outside of the body in catheter draining bag (Govindji, 2013). The urinary catheter (Foley) was created by Fredrick Foley in the 1930s (Lawrence and Turner, 2005). It may be used transiently or for long time. It concerned in 80% of urinary tract infections (Govindji, 2013) when inserted in the body may directly acquire biofilm which form on the inner or outer catheter surfaces (Donlan, 2001). Many microorganism associated with urinary catheter biofilm are Coagulase Negative Staphylococci,

Staphylococcus epidermidis, *Enterococcus* sp., *E. coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and others (Kokare *et al*., 2009).

Duration of using catheters have effect on acquired biofilm. The longer using and persist the catheter in urinary system for long time have a greater predisposition to biofilm formation(Govindji, 2013). Brisset and coworkers (Brisset *et al*., 1996) found that adhesion of microorganisms on catheter surface depend upon the hydrophobicity between them. Urinary catheter has a hydrophilic and hydrophobic regions on its surface which permit to attach and colonize vast variety of microorganisms. Another factors effect on bacterial attachment and increase it are include elevated urinary pH and divalent cations (Ca^{++} and Mg^{++}). Elevation of urinary pH as a result of produce urease enzyme from bacteria which hydrolyze the urine to ammonium hydroxide (Donlan, 2001), lead to precipitation of minerals and that associated with biofilm formation to cause inner lumen catheter blockage (Tunney *et al*., 1999), and that may be caused kidneys failure lead to death because the urine cannot pass through the catheter and push back to the kidney(Hong *et al*., 2012), bacteria may form biofilm by ascend into lumen of patients bladder through the catheter by possess swarming ability like *Proteus* sp. (Donlan, 2001).

In general biofilm formation on urinary catheter depend on many factors, the patient's immune state, duration of catheter use, the type of catheter (surface and quality) and the bacterial strain present in urine (Wong and Hooton, 2005).

1.2.8.Pathogenesis of urinary catheter associated biofilm:

Urinary tract infection causing by urinary catheter increase in USA and occur at more than one million annually (Tambyah *et al*., 2002). Urinary catheter does not increase the entree of pathogenic microorganisms to the bladder, but it is also ready to prepare a surface to form biofilm (Trautner *et al*., 2005). The pathogenesis is related to contamination of inert catheter with pathogens and colonization occur (Trautner and Darouiche, 2004).

Bacteria when present on mucosa of urinary bladder inducing immune response to influx of neutrophils and discard the epithelial cell with colonization bacteria (Klumpp *et al*., 2001) and that lead to removal of bacteria from bladder mucosal surface while in catheter there is no defense mechanisms (Trautner and Darouiche, 2004). Primarily the initiate of biofilm formation on urinary catheter began when the components of urinary system like electrolytes, protein and organic materials deposit on urinary catheter (Denstedt *et al*., 1998). That make the surface of catheter ready to colonize of pathogenic bacteria, motile bacteria by free swimming motility ascend and attach to catheter surface by electrostatic and hydrophobic interactions via its flagella (Pratt and Kolter, 1998).

After attachment, colonization occur and cell division then aggregate more planktonic bacteria to secret extracellular matrix, formation of biofilm as 3 dimensional structure by produce chemical signaling (quarm sensing) to direct cell to cell aggregation and nutrient and wastes exchange through fluid channels production between them (Kolter and Losick, 1998). Last stage of biofilm is the detachment of

individual microorganisms to complete the cycle and may germinate the urine with pathogens (Trautner and Darouiche, 2004).

1.2.9.Advantages of Biofilms:

Bacterial cell can benefit biofilm case by many advantages. The main ones are antibiotics resistance in addition to disinfectant and antiseptic, evade immune system response and protection from the conditions of environment (Archer *et al.*, 2011).

1.2.10. Resistance of biofilm forming bacteria to antimicrobial agents:

A biofilm is multilayer accumulation of bacterial cells and matrix production which provide a protection from antibiotics action. Sessile bacteria more resistance to antibiotics than planktonic bacteria (Zhang *et al*., 2011).

1.2.10.1. Mechanisms of biofilm-associated antimicrobial resistance:

Bacterial biofilm was resistant to antibiotics according to multifactorial and there are four predominant theories regarding these resistance. The first one is that the EPS provides mechanical shielding from the environment, reducing the exposure of the bacteria to antimicrobial agents in the environment by block antibiotics penetration through multilayers matrix. For example, the EPS appears to protect *P. aeruginosa* against the antibiotic tobramycin (Tseng *et al*., 2013). The second theory is that the EPS maintains a concentrated assortment of antimicrobial peptides within the EPS matrix, such as beta lactamases, thus providing active digestion of antimicrobials, modification of antibiotics by bacterial enzymes and efflux pump to some antibiotics

(Burmølle *et al*., 2014). An experiment supporting this theory showed that extracts of the EPS of *S. epidermidis* interfered with the antimicrobial activity of vancomycin and teichoplanin (Camargo *et al*., 2011).

The third theory is that due to microenvironment represented by low nutrients, oxygen and chemical gradients which lead to low metabolic activity and decrease in cell division rate to result slow growth or no growth convert activity bacterial cell to semi dormant cell (Lewis, 2005, Van Acker *et al*., 2014). The dormancy of bacteria in biofilms made it insensitive to drugs blocking the cell cycle (Fux et al., 2005). Waste production as a result of highly density of biofilm may affect action of antibiotics (Pozo and Patel, 2007).

Another theory at molecular level, the resistant antibiotics of bacterial biofilm may explain the alter in gene expression patterns (Van Acker *et al*., 2014), or horizontal gene transfers between closely aggregate bacterial cell which shearing genes by mobile genetic elements which increase resistance rate of antibiotics (Madsen *et al*., 2012). Some of these theories are shown in (Fig. 1-2)

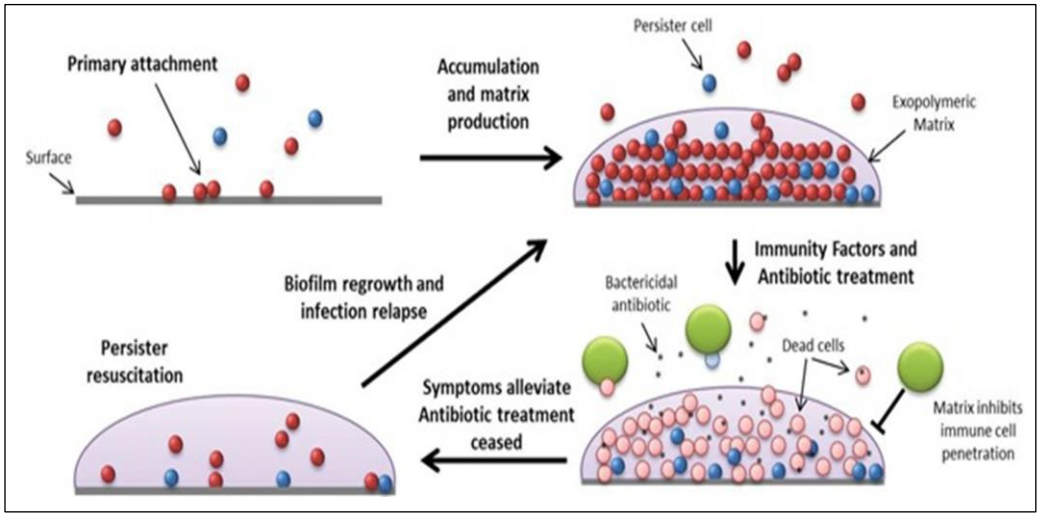


Figure (1-2): Mechanism of antibiotics resistant into biofilm (Wilkinson, 2016).

1.2.11. Role of nanoparticles in medicine:

Nano-science is a science that studies the phenomenon of modulation and fabrication of materials from macromolecules to micromolecules and atomic scale with significant difference properties than large scales (Filipponi and Sutherland, 2013). Nanotechnology is an incorporation of many science fields which promising platform in medicine and pharmaceutical industry (Mohanpuria *et al*., 2008) in addition to computers, electronics, and imaging technology (Morris and Willis, 2007). The physicist Richard Feynman were the first one introduce the nanotechnology world in 1959 when he said in famous lecture "There's Plenty of Room at the Bottom" (Feynman, 1960), which he suggested the possibility to manipulate matter to atomic scale, then he made large revolution in nanotechnology field (Khan *et al*., 2017).

Concerning biomedical biofilms, nanotechnology is emerging as one of the most auspicious methodologies for its prevention and control. The main nano approaches that have demonstrated the most promising results include: silver nanoparticles, drug delivery nano carriers or phage therapy (Sousa *et al*., 2011).

Nanoparticles is a small particles that have range of size 1-100 nm (nm=10^{-9} m) (Laurent *et al*., 2008). It has a novel or unusual properties than their bulk minerals counterparts and act as union part (Daniel and Astruc, 2004, Kato, 2011). As a result of break down the materials to tiny particles, the surface area increases dramatically (Lin *et al*., 2014). Highly surface area and nano-size features make nanoparticles possess unique chemical and physical properties and more reactive chemically by providing a greater reaction surface than their large scale which facilitate interaction and puncturing micrometer sized bacterial cell membranes by

provides a great driving force for diffusion without doing harm to larger host cells (Sass, 2007, Tran and Tran, 2012, Khan *et al*., 2017). According to these characteristics, nanoparticles paying attention to suitable candidates for various application.

The antimicrobial properties of nanoparticles provide an alternative to antibiotics, without a significant risk of resistance mutations. This is significant to consider the development of new antimicrobials which have been relatively unsuccessful (Pompilio *et al*., 2012). It is presently well established that nanoparticles are better than microparticles at resisting biofilm formation (Raghupathi *et al*., 2011). Nanoparticles may be delivered free floating in nanoparticle gels or suspensions, as particles designed to elute from a surface or bound to a nano-textured surface. The shape of nanoparticles has an important role to determine their surface area, for example the spherical shape of nanoparticles has surface area as smallest one. In addition to shape, the size of nanoparticles also effects the surface area measurement so the change in these two factors will affect physiological and physiochemical characterization (Lin *et al*., 2014).

There are many examples of nanoparticles such as: carbon nanorods, carbon nanotubes, titanium oxides, platinum NPs, gold NPs, magnetic NPs and silver NPs … etc. (Duncan and Gaspar, 2011, Mahajan *et al*., 2013).

1.2.12. Applications of NPs in medication fields:

Nanoparticles have special and superior properties to make it suitable candidate for various applications such as medicine, pharmaceutical industry, cosmetic, computers, electronics, and imaging technology… etc. (Khan *et al*., 2017).

Nanoparticles play an important role in medication, biomedical, biological and pharmaceutical field by using as novel antimicrobial, drug delivery (Loureiro *et al*., 2016). Some of uses of nanoparticles in this field are early diagnostic of diseases which also can be used as nutrient level determination in the body. In addition to use as therapeutic agents duo to their antibacterial properties as dressing wound, covering catheter and antimicrobial agents (AshaRani *et al*., 2008) and that most important aspect of nanoparticles application. Example of nanoparticles are Ag, Au, Ti, Zn, Cu- and Ni NPs.

1.2.12.1. Nanoparticles as antimicrobial agents:

At present day, antibiotic resistant bacteria increase and become a serious problem to the health in the world, among them are biofilm and multidrug-resistant bacteria. So it need to develop a novel effective antibacterial. The evolution of nanoparticles create a new antibacterial options and a promising platform to control bacterial diseases as alternative medicine. Nanoparticle is very suitable candidate to carry out antimicrobial operation, as a result to its small size (Beyth *et al*., 2015).

1.2.12.1.1. Silver as an antimicrobial agent:

In the past history, silver was used as antibacterial. The Romans used it as wound dressing while in antiquity especially the Phoenicians used in made the cutlery and vessels to store the water. It used also in medicine as 1 % in concentration of silver nitrate to prevent the infection of eye in newborn children (Filipponi and Sutherland, 2013).

During the second world war, the silver nitrate used directly in healing the wound (Atiyeh *et al*., 2007, Law *et al*., 2008). The food and drug administration (FDA) in USA in 1920s approved for the first time to

use the silver nitrate in management the wound. Until the antibiotics discover, silver were added to different cream for wound healing (Rai and Duran, 2011) in addition to germicides, antiseptics and disinfectant (Filipponi and Sutherland, 2013), and even in cosmetic products (Silver *et al*., 2006), then combination occur between silver and antibiotics to be useful in wound healing, silver nitrate combine with sulfadiazine as cream available in trade mark 'Silvazine' which had a greater action than antibiotic alone (George *et al*., 1997). Moreover it increases bacterial susceptibility to antibiotics when combined with them as synergistic effect especially in biofilm infection like nitrofurazone increased its effect in silver present (Kostenko *et al*., 2010).

Silver having long standing antibacterial compound and silver nanoparticles are more potent in antimicrobial effect than normal scale (Sass, 2007). In case of biofilm caused by urinary catheter, a main challenge is to treated by silver nanoparticles because it involves a vastly mixed resistant microorganisms, even it may display resistance to heavy metal (Woods *et al*., 2009). So one of the successful way to prevent biofilm formation on urinary catheter is by coating the catheter with silver nanoparticles, also it is active against nosocomial infection associated with catheter. Silver nanoparticles in addition to protective aspect, there is no record a risk to systemic toxicity (Roe *et al*., 2008).

Silver nanoparticles when act as antibacterial, there are many mechanism to do the action such as binding to prokaryotic DNA to loses its structure and inhibiting its replication and may also binding to thiol group in protein to make it functionless. Via producing free highly reactive oxygen radicals lead to cell destruction by induction synthesis of reactive oxygen species by Inhibit NADH dehydrogenase II enzyme in respiratory system (Matsumura *et al*., 2003).

1.2.13. Characterization of NPs:

Nanoparticle have many properties include: size, surface properties, shape, solubility, composition, aggregation, molecular weight, identity, zeta potential, purity and stability, are associated with physiological interactions (Amiji, 2006), and that's may offer profits in medical applications, such as decreasing in side effect, efficacy improvement and treatment (Farokhzad and Langer, 2006). To evaluate these properties there is several techniques some of them are; optical spectroscopy, zeta-potential measurements, electron microscopy including transmission and scanning (TEM and SEM), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), X-ray diffraction (XRD), nuclear magnetic resonance (NMR) and particle size analysis (Sapsford *et al*., 2011, Khan *et al*., 2017).

Most of nanoparticles properties is influenced by its morphological features. Different techniques used to determine the morphological features of nanoparticles, the most important techniques are SEM and TEM, these technique based on electron scanning and transmission through the nanoparticles sample and provide all information about the shape and size at nanoscale level (Khan *et al*., 2017).

The size of nanoparticles have a crucial role in biomedicine field, in blood stream the size regulate navigation and circulation of NPs, in addition to affect penetration the drug across the physiological barriers, localization on specific site and cellular responses induction (Ferrari, 2008, Jiang *et al*., 2008). Size of the NPs can be estimated by diverse techniques some of them are dynamic light scattering (DLS), Scanning and transmission electron microscope (SEM and TEM), and X-ray diffraction (XRD), last three technique provide better estimate to particle

size (Kestens *et al*., 2016), while the zeta potential size analyzer/ DLS can be measure the NPs size at extraordinary low level (Khan *et al*., 2017).

The shape of nanoparticles in addition to surface properties and size, play important role in degradation, internalization, targeting, transport and drug delivery (Mitragotri, 2009, Jiang *et al*., 2013). The carrier of drug delivery was influenced by nanoparticles shape also affect its efficiency (Decuzzi et al., 2009), while phagocytosis of microphages to carriers of drug delivery was dependent also on its shape (Champion and Mitragotri, 2009). Furthermore, the antibacterial activity of silver nanoparticles influence by its shape. In Pakistani study, the researcher notices that smaller spherical shape silver nanoparticles are more effective as antibacterial than others shapes, triangular and large spherical(Raza *et al*., 2016).

Most functions of molecular composition and physical structure of nanoparticles surface create surface properties which control the interaction between nanoparticles and its environmental materials (Amiji, 2006). Important parameters of surface properties are surface composition, surface charge, surface energy, surface adhesion and absorbance (Powers *et al*., 2006). The composition of Surface was related to the superficial layers, surface charge affect penetration through physical barriers and on binding to receptor which rule the aggregation of nanoparticles, the charge was estimated by zeta potential, Surface energy is important in aggregation, accumulation and dissolution of nanoparticles (Lin *et al*., 2014).

Different factors influence on NPs stability like temperature, pH, moisture, NPs size, the solvent, exposure to radiation or degradation

enzyme (Briscoe and Hage, 2009). NPs like pharmaceutical product need to maintain the stability for long time. The stability of pharmaceutical product refers to keeping the same properties for a time of period after manufactured (Lin *et al*., 2014).

1.2.14. Synthesis of nanoparticles:

Many methods depend on synthesis nanoparticles but at the general there are two classes to synthesis: top down (physical) and bottom up (chemical and biological) (Wang and Xia, 2004). In top-down class, the broking down of the bulk materials to the nanoparticles contrawise to bottom-up class were the nanoparticles building up to bulk material. Some research thought that bottom-up way is more suitable than of top-down way as a result of chance of contamination occur at high rate (Sharma *et al*., 2017).The physical method is such as irradiation, thermal decomposition method, diffusion method .. etc. while bottom up involves chemical and biological method: reduction and sedimentation techniques; chemical reduction, electrochemical synthesis, sol gel, biochemical synthesis, green synthesis, and spinning (Sahoo *et al*., 2009, Iravani, 2011, Rai and Duran, 2011). All these methods may synthesize nanoparticles as different shapes and sizes. The most chemical method used in nanoparticles synthesis is the chemical reduction which reduce the metal particles to Nano-sized particles by reduction agents such as sodium citrate, sodium borohydride elemental hydrogen, and ascorbate…etc. (Rai and Duran, 2011, Sharma *et al*., 2017) chemical reduction is a most common method for silver nanoparticles synthesis (Sharma *et al*., 2017).

In medical field need to develop ecofriendly, nontoxic nanoparticles synthesis method, the best choice is to use microorganisms

to nanoparticles synthesis which called biosynthesis. Although the chemical method produce a large quantity of nanoparticles in a short time with specific size and shape but its costly, complicated, non-efficient and the most important aspect is may contain a toxic materials that harmful to health of human. In contrast to biological method were more acceptable method because its safety for environment and human health (Li *et al*., 2011).

Many microorganisms like Bacteria (Shahverdi *et al*., 2007, Husseiny et al., 2007), Fungi (Govender *et al*., 2009, Kumar *et al*., 2007), Lichens (Shahi and Patra, 2003), Actinomycetes (Ahmad *et al*., 2003), algae (Singaravelu *et al*., 2007) etc.. can produce nanoparticles through two routs; intracellular and extracellular (Shaligram *et al*., 2009, Li *et al*., 2011). The intracellular rout: the filtrate of bacterial cell deal with metal salt then kept in a shaker incubator with dark condition (Mouxing *et al*., 2006). The extracellular rout: by using bacterial supernatant after centrifuged at 8000 rpm then deal with metal salt, and incubate in dark condition (Ogi *et al*., 2010).

In principle, the microorganism can synthesize nanoparticles by redoxing enzymes which are produced by bacterial activities, then act as electron shuttle to snatch the target ions from its environment to reduce the metal ion to nanoparticles (Sadowski *et al*., 2008, Li *et al*., 2011), which lead to precipitate the product nanoparticles on cell external environment (Sadowski *et al*., 2008). In biological synthesis method the protein responsible for ion reduction found to secret at a large amount(Thakkar *et al*., 2010).

Nitrate reductase in bacteria is the main enzyme concerned on silver nanoparticles synthesis (Kalimuthu *et al*., 2008). The reduction of

this enzyme as a-NADPH dependent nitrate reductase metal ion (Ag^+ ions) to synthesis nanoparticles (silver NPs), that enzyme act to convert the nitrate to nitrite (Durán *et al*., 2005). Then shuttled the electron to silver ions (Rai and Duran, 2011). In 1984 it was the first time to find bacteria isolated from silver mines, produce silver NPs by *Pseudomonas stutzeri* AG259 at size range 35-46nm were accumulated in priplasmic space (Haefeli *et al*., 1984) then that opened a new ways to prepare a nanoparticles from bacteria.

Numerous bacterial strain could synthesis of silver nanoparticles at various range of size like *Lactobacillus* 20 nm, *Bacillus licheniformis* 50 nm, *E. coli* 50 nm, *Corynebacterium glutamicum* 5-50nm, *Bacillus cereus* 4-5 nm spherical and others (Li *et al*., 2011).

Chapter Two

Materials and Methods

2. Materials and methods

2.1. Materials:

2.1.1. Patients: A total of 65 catheterized inpatients at different age groups and both sexes were enrolled in this study whom hausted in different departments of four Iraqi hospitals (AL- Diwaniya Teaching, AL- Hilla Teaching, AL- Qasim and AL- Hashimiya Hospitals) through a period from February to April 2017, suffering from different diseases and clinical cases. A questionnaire form was made to take full history from patients about (age, duration of catheter, clinical cases associated and administration of antibiotics) (Appendix I).

2.1.2. Equipments and Instruments:

The equipments and instruments used in current study are listed in (Table 2-1)

Table (2-1) : **Equipment and instruments:**

Equipment and Instruments	**Manufacture (country)**
Cold centrifuge	Hettich (Germany)
Density Checker™	Biomerux (France)
Fourier Transform Infrared Spectrometer (FTIR)	Bruker Tensor (Germany)
Gel electrophoresis	Bioneer (Korea)
Laminar flow cabinet	Safemate 1.2 (USA)
Laser Particle Size Analyzer	Angstrom Advanced Inc (USA)
Light Microscope	Olympus (Japan)
Micro ELISA auto reader	Dynatech Mr580 (USA)
Nanodrop	Thermo Scientific/ (UK)
PCR Thermocycler	MyGene, Bioneer. (Korea).
PH-meter	Jenway (UK)
Real time PCR	Bioneer (Korea)
Scanning Electron Microscope (SEM)	Inspect S50/FEI (Netherland)

Shaking incubator, Sonicator water bath	Lab Tech (India)
Tissue culture plates (TCP)	China
Ultraviolet transilluminator	DIAHAN (Korea)
UV. visible spectrophotometer	SPEKOL 1300 (Germany)
VITEK system	bioMerieux (France)
Vortex	Stuart (UK)

2.1.3. Chemicals and Biological Materials:

The chemicals and biological materials were used in this study are listed in (Table 2-2)

Table (2-2): Chemicals and biological materials with their remark:

Chemicals &Biological Materials	**Manufacture (country)**
Acetone, Chloroform, Ethanol (95%), Gram stain set, Sodium acetate, Sodium borohydride, Sodium chloride (NaCl)	BDH (UK)
Agarose, TBE buffer	Condalab (Spain)
AgNO3, Congo red stain, Ethidium bromide, Kovac's reagent, Methyl red	Sigma Aldrich (USA)
Azithromycin (200mg/5ml)	Riva pharma (Egypt)
Crystal violet	Sorachim (Switzerland)
Deionized water, DNA marker Ladder	Bioneer (korea)
Glucose	Merck
Glycerol	Fluka (Germany)
Impienem vial (500)	Merck sharp (USA)
Pepton	Oxoid (England)
Phosphate buffer saline	BioBasic (Canada)
Sterile slain (0.45-0.50%)	Chain
Sucrose	Thomas baker, India

2.1.4. **Culture media:** The following culture media were used properly in appropriate experiment:

Table (2-3): Bacterial culture media used with their remarks:

Culture media	Manufacture (country)
Agar agar, Blood agar, Brain heart	Himedia (India)

infusion agar, Brain heart infusion broth, Carbohydrate fermentation media, Eosin methylene blue, MacConkey agar, Muller Hinton agar, Muller Hinton broth, Nutrient agar, Nutrient broth, Trypticase soy broth,	

2.1.5. Biochemical diagnostic kit(VITEK system):

VITEK kit were used to diagnostic bacterial isolates are listed in (Table 2-4).

Table (2-4): Diagnostic Kit with its manufactured country:

Kit	Purpose	Manufacture Country
VITEK®2 GP	Gram-Positive Identification	Biomerieux – France
VITEK®2 GN	Gram-negative Identification	
VITEK AST	*Staphylococcus* sp.	
VITEK AST	Gram negative	

2.1.6. Molecular Diagnostic Kits:

The molecular diagnostic kits with their company are listed below in table(2-5) and (2-6).

Table (2-5): Molecular diagnostic kit of PCR:

No.	Kit	Manufacture(Country)
1	**Genomic DNA Extraction Kit**	**Geneaid** (USA)
	GT buffer	
	GB buffer	
	W1 buffer	

	Wash buffer	
	Elution buffer	
	GD column	
	2ml Collection tubes	
2	**AccuPower® PCR PreMix**	**Bioneer (Korea)**
	Taq DNA polymerase	
	dNTPs (dATP, dCTP, dGTP, dTTP)	
	Tris-HCl pH 9.0	
	KCl	
	$MgCl_2$	
	Stabilizer and Tracking dye	

Table (2-6): Molecular diagnostic kit Real time PCR:

No.	Kit	Manufacture(Country)
1	**Total RNA Extraction Kit** (AccuZol ™)	Bioneer (Korea)
	Trizol reagent 100ml	
2	**DNase I enzyme kit**	Promega (USA)
	DNase I enzyme	
	10x buffer	
	Free nuclease water	
3	**AccuPower® Rocket Script™ RT PreMix 96 plate**	Bioneer (Korea)
	RocketScript Reverse Transcriptase (200 u)	
	X Reaction Buffer (1 x 5)	
	DTT (0.25 mM)	
	dNTP (250) µM each	
	RNase Inhibitor (1 u)	
4	**AccuPower® Green star™ qPCR PreMix 96 plate**	Bioneer (Korea)
	SYBER Green fluorescence	
	Taq DNA polymerase	
	dNTPs (dATP, dCTP, dGTP, dTTP)	
	DEPC water	

2.1.7. Primers

The PCR and Real Time PCR primers were same which designed using NCBI Gene-Bank and Primer 3 online. These primers were provided by (Macrogen. company, Korea) (Table2-7 and 2-8):

Table (2-7): PCR primers and their sequence with Gen Bank codes:

Primer	Sequence 5'-3'		Amplicon	Bacteria
icaA gene	F	TGGATGTTGGTGCCTGAAAC	77bp	*Staphylococcus* sp.
	R	AGTACTTCATGCCCACCTTGAG		
esaI gene	F	TTTTGCCACCGCGTCAAAAC	127bp	*Pantoea* sp.
	R	TGGCGTATCGTTGCTGAATC		
smaI gene	F	TCACGTCATTTGCAGCTTGC	73bp	*Serratia* sp.
	R	ATTGTTGAACACGCCATCGC		

Genbank: *icaA*: DQ836167.1, *smaI*: AM236917.1, & *esaI*: AY876938.2

Table (2-8): Real-Time PCR Primers:

Primer	Sequence 5'-3'		Amplicon
S. lentus-rpoB	F	TGCTTGACGTTGCATGTTCG	83bp
	R	CTGCACCATCAGCATGTATTCC	
Biofilm- *S. lentus* (*icaA*)	F	TGGATGTTGGTGCCTGAAAC	77bp
	R	AGTACTTCATGCCCACCTTGAG	
Pantoea-rpoB	F	ATCAACGCCAAGCCCATTTC	101bp
	R	TGCGTAATCTCTGACAACGG	
Biofilm – *Pantoea* sp. (*esaL*)	F	TTTTGCCACCGCGTCAAAAC	127bp
	R	TGGCGTATCGTTGCTGAATC	
S. fonticola -rpoB	F	TCACGCACAAACGTCGTATC	79bp
	R	ACGTCTCGAACTTCAAAGCC	
Biofilm- *S. fonticola* (*smaI*)	F	TCACGTCATTTGCAGCTTGC	73bp
	R	ATTGTTGAACACGCCATCGC	

2.1.8. Antibiotics disc: the antibiotics disc which used in antimicrobial susceptibility testing (disc diffusion method) are listed (Table 2-9).

Table (2-9): Antibiotics disc with potency and Manufacture company:

Antibiotics	Symbol	Potency	Manufacture
Amikacin	AK	30μg	Bioanalysis, India.
Azithromycin	AZM	15μg	
Carbencillin	PY	100μg	
Ciprofloxacin	CIP	10μg	
Clindamycin	DA	2μg	
Gentamicin	CN	10μg	
Imipenem	IPM	10μg	
Novobiocin	NV	5μg	
Penicillin G	P	10 IU	
Tobramycin	TOB	10μg	
Oxacillin	OX	1μg	
Vancomycin	VA	10μg	

2.1.9. DNA marker:

Table (2-10) : Accupower molecular weight DNA marker:

DNA ladder	Description	Source
50bp and 100bp	50 -2000base pairs and 100-2000 base pairs. The ladder consist of 18 double strand DNA fragments ladder with size of (50, 100,150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000)	KAPA- South Africa

2.1.10. Reagents and Solutions :

2.1.10.1. Gram Stain Solution:

Gram Stain was provided by BDH company. This solution often uses for studying the cell morphology and arrangement of bacterial cells (Forbes *et al*., 2007)

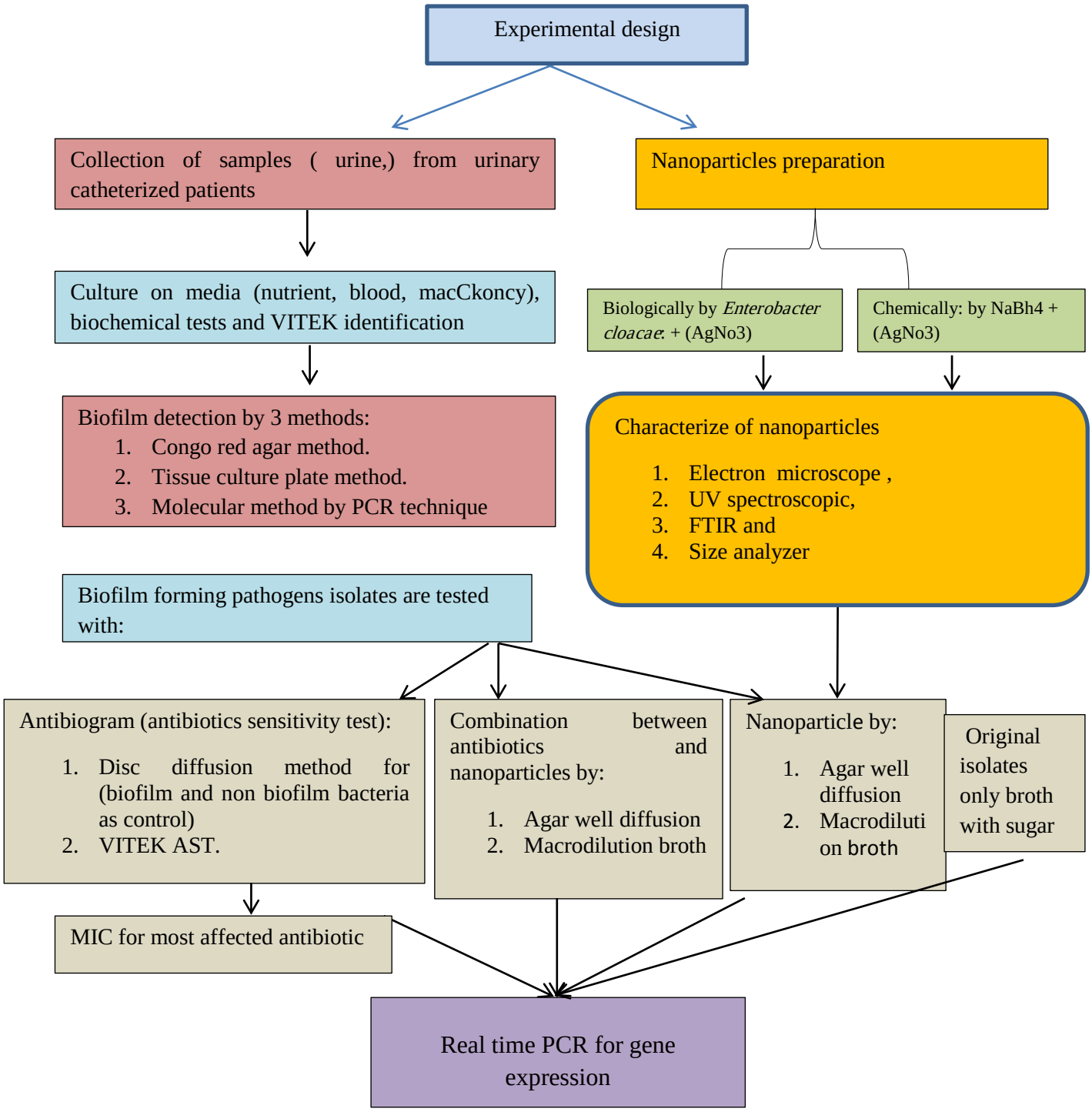
2.1.10.2. Phosphate Buffer Saline (PBS):

One tablet of PBS (pH 7.3) was dissolved in 100 ml of D.W to prepare this solution, the resulting solution was sterilized by autoclave (Sambrook *et al*., 2001).

2.1.10.3. 1X Tris Borate EDTA (TBE) Buffer:

1X Tris Borate EDTA (TBE) Buffer were prepared by diluting the concentrated TBE buffer (10X). It was used to dissolve agarose and in electrophoresis procedure. 100 ml of TBE (10X) was added to 900 ml of D.W to reach to 1X TBE concentration (Sambrook *et al*., 2001).

Study design: The current study was designed as following diagram.



2.2. Methods:

2.2.1. Collection of samples:

The urine samples were collected by straight catheter from 65 catheterized patients in sterile test tubes and transported immediately to laboratory (Forbes *et al*., 2007). All patients had been exposed to antibiotics at least before 3 days.

2.2.2. Culture media:

2.2.2.1. Preparation of the Culture Media:

All the media using in the current study were prepared according to their instruction manufacturing company. It were sterilized by using autoclave at 121˚C for 20 min.. These media were listed in (Table 2-11):

Table (2-11): The Media that used in this study and their purposes:

No.	Media	The purpose of using
1	Blood Agar	Used to determine the ability of bacterial isolates for hemolysis the blood and also it use as an enrichment medium (Forbes *et al*., 2007).
2	Brain Heart Infusion Broth.	Used to activate the bacterial isolates (MacFaddin, 2000).
3	Luria Broth Media	Used for DNA extraction (MacFaddin, 2000).
4	MacConkey Agar	Utilize for the primary isolation of G^{-ve} bacteria and to distinguish between lactose fermenters bacteria non-lactose fermenter (MacFaddin, 2000).
5	Mannitol salt agar	Selective and differential media to *Staphylococcus* (MacFaddin, 2000).

6	Muller-Hinton Agar and Muller-Hinton Broth.	This media used in antimicrobial susceptibility testing (Forbes *et al*., 2007).
7	Nutrient Agar, Nutrient Broth	Subculture and growth of bacterial isolates (MacFaddin, 2000).
8	Peptone Water Medium	Demonstrate the ability of bacteria to decompose tryptophan to indole (MacFaddin, 2000).
9	Tryptic Soy Broth	Used to cultivate the bacterial isolates (Forbes *et al*., 2007)

2.2.2.2. Hemolysin Production:

Hemolysin production bacteria were tested by using 5% blood agar, these isolates were inoculated on blood agar and incubated for 24 hrs. at 37^0C. Hemolysin production was identified by clear zone around the colony which indicate erythrocyte lysis(Forbes *et al*., 2007).

2.2.3. Isolation and identification of bacteria:

Urine samples were cultured in enrichment media (brain heart infusion broth) were incubated at 37^0C for 24hrs. then the bacterial growth was cultured to routinely media on (nutrient, MacConkey and blood agar) were incubate at 37^0C for 24hrs.. The positive growth culture was counted according to the number observed per plate, which consider bacteriuria when it detect as more than 10^5 CFU/ml in urine sample (Forbes *et al*., 2007) then biochemical test, staining and microscopic examination were done (MacFaddin, 2000). Bacterial isolates identified by the automated system VITEK 2 to reach final diagnostic and

identification. The identification with VITEK 2 includes ID-GN card for gram-negative and ID-GP card for gram-positive bacteria.

2.2.3.1. VITEK analysis:

VITEK analysis were processed as the following steps according to instruction of its manufacturer.

2.2.3.1.1. Bacterial suspension Preparation:

A sufficient amount of pure bacterial colonies was transferred by sterile stick to plastic test tube contain 3 ml of sterile saline at concentration (0.45-0.5 % NaCl) to suspended the bacterial cells, the turbidity measured by Density Checker (turbidity meter) adjusted to 0.50-0.63 which equivalent to 1.5 x 10^8 CFU/ ml to both Gr^+ and Gr^- (Pincus, 2006).

2.2.3.1.2. Bacterial inoculum:

Bacterial cell suspension were placed in plastic test tubes into a special rack while identification cards or cassette placed in apposite slot then the transfer tube transferred the suspension bacterial cell to identification cards to fill all tests wells, the cards contain up to 15 tests. After that the transfer tube was cutoff to seal the cards and to load into interior incubator at 37 c for 24 hrs. (Ligozzi *et al*., 2002, Pincus, 2006).

The results read digitally on monitor connected to VITEK system apparatus.

2.2.3.2. Reference strain:

Serratia marcescens and *E. coli* were diagnostic by biochemical tests as reference strain to *Serratia fonticola* and *Pantoea* sp. respectively.

Staphylococcus control sp. were diagnostic by Novobiocin as table below according to (CLSI, 2017).

Table (2-12): Novobiocin susceptibility to *Staphylococcus* sp.:

Antibiotic	*S. aureus*	*S. epidermidies*	*S. saprophyticus*
Novobiocin	Sensitive	Sensitive	Resistant

2.2.3.3. Biofilm detection: To detect biofilm forming bacteria three methods were done as follow:

2.2.3.3.1. **Congo red agar method:**

Brain Heart Infusion Broth, agar agar supplemented with 50gm/l sucrose and 8gm/l Congo red (Freeman *et al*., 1989). To obtain Congo red agar we prepared a Congo red stain as stock solution, autoclaved at 121°C for 20 min then added to autoclaved brain heart infusion broth with agar agar and 5% sucrose at 55°C.(Hassan *et al*., 2011). The bacterial strains were inoculated and incubated at 37 °C for 24 to 48 hrs. then read the result as following: if the bacteria formed black colonies with a dry crystalline consistency that was mean it biofilm producer isolates while if it formed red colonies that was mean the non-biofilm producer isolates (Kaiser *et al*., 2013).

2.2.3.3.2. **Tissue culture plate method:**

Tissue Culture Plate (TCP) assay firstly described by (Christensen *et al*., 1985) was used to detect biofilm formation but with some modification.

Tryptic soy broth(TSB), 1% glucose(w/v),phosphate buffer slain (PBS) pH 7.2, crystal violate, 2%sodium acetate and ethanol 95%. Micotiter plate with cover and micoplate reader

1) Bacterial strain was inoculated in autoclaved TSB then incubated at 37°C for 18hrs.
2) The bacterial culture was diluted 1: 100 in TSB supplemented with 1% glucose then transferred 200 µl from diluent culture to each well in 96 well-flat bottom tissue culture plates which performed by triplicate (3 wells for each sample) only broth (TSB) served as control, incubate at 37°C for 24hrs.
3) Supernatant were removed and washed three time with PBS.
4) The plate was dried at room temp. then fixed by 2% sodium acetate.
5) Then it was stained with 0.1% crystal violate for 10 min. and washed by deionized water.
6) Ethanol 95% was added at 200 µl for 10min. to dissolve bound crystal violet.
7) The optical density (O.D) value was measured at 630nm by microtiter plate reader.

The mean of absorbance value from replicated wells were read and the biofilm degree calculate according to this equation:

Biofilm degree =Mean OD_{630} of tested bacteria- Mean OD_{630} of control

The results were interpreted according to the following Table (2-13):

Table (2-13): Classification based on OD values

Mean OD value	Adherence	Biofilm formation
<0.120	Non	Non/ weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	Strong

Modified TCP method was considered as gold standard (Bose *et al*., 2009).

2.2.3.3.3. **Molecular method:**

PCR and Real Time PCR technique were performed for Biofilm formation genes (*icaA*, *smaI* and *esaI* genes) for *Staphylococcus* sp., *Serratia* sp. and *Pantoea* sp. respectively as following steps:-

(1) **Bacterial DNA extraction method**: to extract bacterial nucleic acid, the method was done according to manufacture protocol of extraction method (Geneaid, USA).

(2) **Nanodrop:** DNA extraction product was estimated at 260/280nm by nanodrop device then kept at deep freezer for used later in PCR analysis.

(3) **Primers**: The selected primers were designed by NCBI Gene sequence data base using and primer 3 plus design as table (2-7):

(4) **Master mix preparation**: by using master mix reagents were done according to instruction of company as following table:

Table (2-14) PCR master mix instructions company for each gene:-

PCR master mix	Volume
DNA template	5µL(50ng)
Forward primer (10pmol)	1.5µL
Reverse primer (10pmol)	1.5µL
PCR water	Up to 20µL

Then, the PCR master mix that prepared as table above placed in AccuPower PCR -PreMix that contain all other PCR components such as (Taq DNA polymerase, dNTPs, PCR buffer). Then, PCR tubes were placed into centrifuge for 3 min., after that they transferred into PCR thermocycler apparatus.

2.2.3.3.3.1. **PCR thermocycler conditions:**

Table (2-15) the conditions of PCR thermocycler:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	30 sec	30
Annealing	58 °C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	5 minute	1
Storage	4°C	Hold	

2.2.3.3.3.2. **PCR product analysis:**

The products of PCR were analyzed by electrophoresis in a 1% agarose gel at following steps:

1- Agarose gel at 1% was prepared in using 1X TBE and milted in microwave, then left to cool at 50°C. Ethidium bromide stain(10mg/ml) were added at 3 µl into agarose gel solution.

2- The comb was fixed in proper position of tray and agarose gel solution was poured in it then left to solidified for 15 minutes at room temperature, after that the comb was removed gently from the tray and the PCR product were added in 10µl to each comb well and 5ul of (50 and 100bp Ladder) in one well.
3- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 am.
4- PCR products were visualized under UV-transilluminator, then pick up a picture to bands of interest by digital camera.

2.2.4. Antibiotics susceptibility tests: it was done by two methods

2.2.4.1. Disc Diffusion Method: according to (CLSI, 2015).

The test done on muller hinton agar with antibiotics disc with following steps:

2.2.4.1.1.Preparation of bacterial inoculum:

In disc diffusion method, experimental biofilm forming bacterial isolates (*Serratia fonticola*, *Pantoea* sp. and *Staphylococcus lentus*), prepare its inoculum from overnight bacterial growth in shaker incubator at 150 rpm, 37°C, by selected 3-5 identical colonies by sterile loop to suspend in 3 ml normal slain tube to check the density of cell by density checker apparatus at 0.5 which equivalent to McFarland turbidity standard at 1.5×10^8 CFU/ml.

Macrodilution method was used to determine the MIC value, the bacterial strain culturing in Muller Hinton broth with 1% glucose incubated overnight in shaker incubator at 150 rpm (to induce biofilm gene expression) then diluent the bacterial broth to reach the turbidity

equivalent to 0.5 McFarland standard ($1.5x\ 10^8$) by using density checker apparatus.

2.2.4.1.2.Inoculation of testing plate:

Muller Hinton agar plate were inoculated with bacterial suspension Within 15 min. after chick the density of bacterial suspension, by dipping sterile cotton swab into bacterial suspension then squeezed on tube wall to eliminate the excess amount of inoculum then streaking the swab over the plate surface with repeated the streaking and rotated the plate at 60◦ each time to ensure an even distribution of inoculum. The inoculum plate were left to dry at room temperature for 5 min. before deal with it.

2.2.4.1.3.Antibiotics disc plating:

The selected antibiotics disc were placed on the surface of inoculated plate with tested bacteria by using sterile forceps with appropriate distance between each disc, no more than 8 discs. Then incubation at 37°C for 18-24hrs.

After incubation time, each plate were examined by measured the diameter of complete inhibition zone to the nearest whole millimeter, using a ruler then interpreted the size of inhibition zone measuring according to standard table in CLSI (CLSI, 2017) for Enterobactericeae and *Staphylococcus* sp. respectively as tables below, the bacteria were reported as either susceptible, intermediate or resistant.

Table(2-16): Zone diameter of inhibition in disc diffusion method (CLSI, 2017).

Antibiotics	Enterobactericeae IZ (mm)			*Staphylococcus* sp. IZ (mm)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Penicillin G				≥29	____	≤28
Carbenicillin	≥29	____	≤28			
Imipenem	≥23	20-22	≤19			
Ciprofloxacin	≥21	16-20	≤15	≥21	16-20	≤15
Clindamycin	____	_____	____	≥21	15-20	≤14
Azithromycin	≥13	_____	≤12	≥18	14-17	≤13
Tobramycin	≥15	13-14	≤12	≥15	13-14	≤12
Gentamicin	≥15	13-14	≤12	≥15	13-14	≤12
Amikacin	≥17	15-16	≤14	≥17	15-16	≤14

IZ: inhibition zone

2.2.4.2. Minimum inhibitory concentration (MIC) method:

The test done by two ways.

1) **VITEK 2 AST system**: it were tested to many antibiotics as table (2-17) and (2-18) for *Staphylococcus* sp. and Enterobactericeae respectively. All the following steps were done according to the manufacturer's instructions as diagnostic VITEK 2 system but with antimicrobial susceptibility testing cards for *Staphylococcus* sp. and Enterobactericeae contain more than 15 antibiotics as tables (2-17 and 2-18).

The results read also digitally on monitor connected to VITEK system apparatus.

Table (2-17): Antibiotics provide by VITEK AST card for *Staphylococcus* sp. with MIC breakpoints:

Antimicrobial	MIC Breakpoints (µg/mL)			Antimicrobial	MIC Breakpoints (µg/mL)		
	S	I	R		S	I	R
Benzylepencillin	≥0.12	-	≤0.25	Teicoplanin	≥8	16	≤32

Oxacillin	≥0.25	-	≤0.5	Vancomycin	≥4	8-16	≤32
Gentamicin	≥4	8	≤16	Tetracycline	≥4	8	≤16
Tobramycin	≥4	8	≤16	Nitrofurantain	≥32	64	≤128
Levofloxacin	≥1	2	≤4	Rifampicin	≥1	2	≤4
Moxifloxacin	≥0.5	1	≤2	Trimethoprim/Sulfamethoxazole	≥2/38	-	≤4/76
Erythromycin	≥2	4	≤8	Beta-lactamase	Positive or negative		
Clindamycin	≥0.5	1-2	≤4	Cefoxitin screen	Positive or negative		
Azithromycin	≥2	4	≤8				

Table (2-18): Antibiotics provide by VITEK AST card for Enterobactericeae with MIC breakpoints according to M100 (CLSI, 2017):

Antibiotic	MIC Breakpoints (µg/mL)			Antibiotic	MIC Breakpoints (µg/mL)		
	S	I	R		S	I	R
Ampicillin	≥8	16	≤32	Cefepime	≥2	-	≤16
Amoxicillin/ calvulanic acid	≥8/4	16/8	≤32/16	Trimethoprim /Sulfamethoxazole	≥2/38	-	≤4/76
Ampicillin/ salbactam	≥8/4	16/8	≤32/16	Gentamicin	≥4	8	≤16
Piperacillin-tazobactam	≥2/4	4/4	≤8/4	Tobramycin	≥4	8	≤16
Cefazolin	≥2	4	≤8	Ciprofloxacin	≥1	2	≤4
Ceftriaxone	≥1	2	≤4	Levofloxacin	≥2	4	≤8
Ceftazidime	≥8	16	≤32	Nitrofurantain	≥32	64	≤128
Imipenem	≥1	2	≤4				

2) **Macrodilution broth test** : the test done according to (CLSI, 2012) for imipenem and azithromycin were tested to Enterobactericeae (*Serratia fonticola* and *Pantoea* sp.) MIC and *Staphylococcus lentus* MIC, respectively by following steps:

a) Preparation antibiotics stock solution and serial dilution:

Stock solution of both antibiotics and serial dilution were done based on (Table 2-19 and 2-20) of (CLSI, 2017). Each antibiotic was dissolved and diluted according to (Table2- 21).

Table (2-19): Preparation serial dilution for Imipenem :

Step	Con. (µg/ml)	Source	Volume (ml) +	Diluent (ml)PBS	Intermediate Con.(µg/mL)	Final con. at 1:100 (µg/mL)
1	1600	Stock			1600	16
2	1600	Stock	0.5	0.5	800	8
3	1600	Stock	0.5	1.5	400	4
4	1600	Stock	0.5	3	200	2
5	200	Step4	0.5	0.5	100	1
6	200	Step4	0.5	1.5	50	0.5
7	200	Step4	0.5	3	25	0.25
8	25	Step7	0.5	0.5	12.5	0.125
9	25	Step7	0.5	1.5	6.25	0.0625
10	25	Step7	0.5	3	3.125	0.03
11	3.1	Step10	0.5	0.5	1.6	0.015
12	3.1	Step10	0.5	1.5	0.8	0.008
13	3.1	Step10	0.5	3	0.4	0.004
14	0.4	Step13	0.5	0.5	0.2	0.002

Table (2-20): Preparation serial dilution for Azithromycin:

Step	Con. (µg/ml)	Source	Volume(ml) +	Diluent (ml)Muller Hinton broth	Final Con. (µg/mL)
1	5120	Stock	1	9	512
2	512	Step 1	1	1	256
3	512	Step 1	1	3	128
4	512	Step 1	1	7	64
5	64	Step4	1	1	32
6	64	Step4	1	3	16
7	64	Step4	1	7	8
8	8	Step7	1	1	4
9	8	Step7	1	3	2
10	8	Step7	1	7	1
11	1	Step10	1	1	0.5

12	1	Step10	1	3	0.25
13	1	Step10	1	7	0.125

Table (2-21): Solvent and diluent of antibiotics according to (CLSI, 2017):

Antibiotic	Solvent	Diluent
Imipenem	phosphate buffer saline	phosphate buffer saline
Azithromycin	95% ethanol or glacial acetic acid	broth media

b) **Preparation of bacterial inoculum**: mention in section (2.2.4.1.1.)

1. By using 14 sterile test tubes, each tube contain twofold dilution of selected antibiotics as describe in table (2-19 and 2-20).
2. Tested bacteria inoculum were prepared as described above and added 1ml to each tube except one without antibiotic as control.
3. All these tubes were incubated at 37°C for 18-24 hrs. (CLSI, 2012).

MIC is a lowest antibiotics concentration that inhibit completely the bacterial growth and can detected by unaided eye. The turbidity of bacterial growth in tubes containing serial dilution of antibiotics compared with control tube (without antibiotic) and determine the concentration of tube before first one with turbidity to be the Minimum inhibitory concentration.

The results in both methods were regarded as sensitive, intermediate or resistant based on the (CLSI, 2017).

2.5. Silver nanoparticles synthesis: it was synthesis by two methods.

2.5.1. Chemical synthesis method: it was done by following steps:

2.5.1.1. Preparation of Silver Nitrate ($AgNO_3$) solution:

To prepare 0.01 M of $AgNO_3$, dissolved 0.16 gm. in 100ml de-ionized water according to below equation. It was seen that the solution was a clear liquid and shows mild lustrous appearance (Karthik and Radha, 2012).

2.5.1.2. Preparation of Sodium Borohydride ($NaBH_4$) solution:

At same equation above, sodium borohydride was prepared at 0.001M as follow.

Five ml of 0.01M $AgNO_3$ was added dropwise (1 drop per sec.) to 50ml of 0.001M $NaBH_4$ in beaker (250ml) on magnetic stirrer at (400 for 30 min in dark condition then the change in color was noted. The reaction mixture was stirred vigorously on a magnetic stirrer (Rashid *et al*., 2013, Mehr *et al*., 2015) according to equation below:

$AgNO_3 + NaBH_4 \rightarrow Ag + H_2 + B_2H_6 + NaNO_3$

The entire addition process took about 3 minutes, after which the stirring was stopped and the stir bar was removed. Reaction conditions including stirring time and relative quantities of reagents (Rashid *et al*., 2013).

2.5.2. Biological synthesis methods:

Biosynthesis of silver nanoparticles by ***Enterobacter cloaca*** according to (Shahverdi *et al*., 2007) with modification.

2.5.2.1. Preparation of $AgNO_3$ solution:

By using the equation of chemical synthesis, the con. of $AgNO_3$ at 0.1M were calculated as follow:

= 0.1*169.87*100/1000= 1.69 gm. dissolved in 100ml of deionized water

2.5.2.2. Bacterial strain:

Enterobacter cloaca isolate was isolated from urine of catheterized patients and have ability to produce biofilm.

2.5.2.2.1. Preparation of bacterial supernatant:

Sterilizing nutrient broth were inoculated with *Enterobacter cloaca* fresh culture and incubated in shaker incubator at 150 rpm, 37°C for 24 hrs.. After the incubation period the broth culture were centrifuged at 6000 rpm for 15 min. in cold centrifuge (4°C) then the Supernatant drawing by pasture pipet and filtrate by Millipore filter 0.4, after that 0.25ml of 0.1 M AgNO3 was added to 50 ml of (10ml bacterial supernatant and 40 ml deionized water). All that mixture were incubated in shaker incubator at 150 rpm, 37°C for 24 hrs. Then the visual observation of change in color was noted from yellow to brown color.

2.6. Characterization of silver nanoparticles product:

The silver nanoparticles in two methods were characterized by UV. Spectrophotometer, Size analyzer, SEM and FTIR (Gomaa, 2017). All these analyses were carried out at pharmacy and science college, Kufa and Babylon university and veterinary college of Al-Qasim green university.

2.6.1.Measuring the Surface Plasmon Resonance (SPR) by UV–visible spectroscopy:

The SPR of silver nanoparticles in two methods were measured by UV–visible spectroscopy at wave length ranging from 300-500 nm. By sampling 1ml of SNPs solution to different wave length were measured

every ten degree at resolution of 1nm. only one plasma band is obtained and the increase of its intensity is an indication of the reaction advance degree with subsequent increment in the number of particles (Karthik and Radha, 2012).

2.6.2.Size analyzer:

Laser diffraction particle size analyzers, which measure light scattering and assume an index of refraction to calculate the particle size distribution. It exploited the Mie theory of light, which relates the scattering pattern produced as light passes through a sample to the size of any particles present (Levoguer, 2013). Silver nanoparticles sample was incubated in sonicator water bath at 35C for 30 min. to prevent aggregation of particles before examination in size analyzer. By using laser beam scattering in beta sizer apparatus, emulsion of silver nanoparticles sample were diluted by deionized water then putted in grove in apparatus and the size were measured during 5 min.. The results were monitoring as screen of computer.

2.6.3.Fourier transform infrared spectroscopy (FTIR):

FTIR used to detect the possible present of biomolecules may be associated with SNPs formation in both methods. The powder SNPs sample was measured by mixed with KBr disk at high pressure in wavenumber range of 4000-500 cm^{-1}. Dried SNPs sample was prepared by centrifugation at 10000 rpm for 15 min., the solid pellet was washed by deionized water three times to eliminate any unattached molecules then dried at 40°C before using in FTIR analysis (Gomaa, 2017).

2.6.4.Scanning electron microscope analysis (SEM):

The chemical and biological synthesis SNPs were evaluated their size and shape by using SEM examination. A drop of SNPs solution was placed on the carbon and kept until sample gets dried before loading them onto a specimen holder. The micrographs were taken by analyzing the prepared grids at a voltage of 5-10 kV at different magnifications with low vacuum, a spot size 4 and working distances 5-10mm (Umoren *et al*., 2014).

2.7. Antimicrobial susceptibility assay of silver nanoparticles:

Antimicrobial activity of the chemo and biosynthesized SNPs was evaluated using the agar well diffusion and MIC method.

2.7.1.Agar well diffusion method: it was done as following steps:

2.7.1.1. Preparation of SNPs solution at different concentration:

The chemical synthesis SNPs was prepared as different concentration (170, 150, 130, 110 and 90µg/ml) while the biological synthesis SNPs as (85, 65, 45, 25 and 5µg/ml).

2.7.1.2. Preparation of bacterial inoculum:

Same as mention in section 2.2.4.1.1.

The selected bacterial inoculum seeded on muller hinton agar plate as section (2.2.4.1.2.) then waited 10 min to dry the streaking broth on agar plate to made later 5 wells by cork borer at 8mm. The wells filled with 100 µl from chemical synthesis SNPs solution at different concentrations (170, 150, 130, 110 and 90µg/ml) to each well .

The biological synthesis SNPs was dealt with same procedures above but with different concentrations (85, 65, 45, 25 and 5µg/ml) also filled with100 µl in each well.

Both methods were compared with related bacterial reference strains as control. All plates incubate at 37°C for 24 hrs. with dark conditions. Both SNPs types diluent by deionized water (Gomaa, 2017).

After incubation time, each plate was examined by measured the diameter of complete inhibition zone to the nearest whole millimeter, using a ruler then record the results value.

2.7.2.Minimum inhibitory concentration (MIC): the test was done by using macrodilution method:

Serial dilution of SNPs was made to distribution on 5 test tubes at 3ml with 1ml bacterial inoculum after turbidity checker with control tube (only bacterial inoculum without SNPs solution). The chemical SNPs concentrations were (170, 150, 130, 110 and 90µg/ml) while biological SNPs concentrations were (85, 65, 45, 25 and 5µg/ml). incubated all these tubes at 37°C for 24 hrs. with dark conditions.

MIC is a lowest silver nanoparticles concentration that inhibited completely the bacterial growth and can detected by unaided eye. The turbidity of bacterial growth in tubes containing serial dilution of SNPs compared with control tube (without SNPs) and determined the concentration of tube before first one with turbidity to be the Minimum inhibitory concentration.

2.8. Combination of chemo and biosynthesized SNPs with tested antibiotics

: The test was done according to (Verma, 2007).

2.8.1. Combination between SNPs with Imipenem and SNPs with azithromycin:

To determine the synergism effect by combination between chemical and biological synthesis of SNPs with Imipenem and chemical and biological synthesis of SNPs with azithromycin by two methods, agar well diffusion and macrodilution broth according to (Verma, 2007). In this experiment, same concentration of SNPs used in antimicrobial susceptibility with above and below of antibiotics MIC concentrations were mixed together in both methods (well diffusion and macrodilution broth methods).

2.8.1.1. Agar well diffusion method:

2.8.1.1.1. SNPs with Imipenem:

Each plate containing Muller Hinton agar was inoculated with checker gram negative bacterial inoculum on its surface by same way of streaking in disc diffusion method and by cork borer made a wells each well contain 100 µl from both (50 µl from each one) Imipenem concentration at (8, 4, 2, 1, 0.5 and 0.25 µg/ml) with chemical SNPs concentration at (170, 150, 130, 110 and 90µg/ml) to be (170/8, 150/4, 130/2, 110/1, 90/0.5 and 70/0.25) and with biological SNPs concentration at (85, 65, 45, 25 and 5µg/ml) to be (85/8, 65/4. 45/2. 25/1. 5/0.5).

2.8.1.1.2. SNPs with azithromycin:

Each plate containing Muller Hinton agar was inoculate with checker *S. lentus* inoculum on its surface by same way of streaking in disc diffusion method and by cork borer made a wells each well contain 100 µl from both (50 µl from each one) azithromycin at (512, 256, 128, 64 and 32 µg/ml) with chemical SNPs concentration at (170, 150, 130,

110 and 90µg/ml) to be (170/512, 150/256, 130/128, 110/64 and 90/32 µg/ml) and with biological SNPs concentration at (85, 65, 45, 25 and 5µg/ml) to be (85/512, 65/256, 45/128, 25/64, and 5/32µg/ml). All these plates were incubated at 37°C for 24 hrs. with dark conditions.

After incubation time, each plate was examined by measured the diameter of complete inhibition zone to the nearest whole millimeter, using a ruler then record the results.

Calculate synergistic effect:

The calculation of synergistic effect according to this equation (Verma, 2007) is:

$$\text{Synergistic effect} = \frac{B-A}{A} * 100$$

A= ZOI for antibiotic

B= ZOI for antibiotics + Ag-NB

2.8.1.2. Macrodilution broth method:

After determine the MIC value to antibiotic alone and SNPs alone (both types), the concentration above, equal and below MIC value were used together in this experiment as describe below (dark field was selective con.) (Verma, 2007).

B \ A	1/8 MIC	1/4 MIC	1/2 MIC	MIC	2X MIC
1/8 MIC					
1/4 MIC					
1/2 MIC					
MIC					
2X MIC					

2.8.1.2.1. Combination chemical SNPs with Imipenem:

1. 0.5 ml of Imipenem concentration (8, 4, 2, 1, 0.5 and 0.25 µg/ml) was added to test tubes.
2. 0.5ml of chemical SNPs concentration (170, 150, 130, 110 and 90µg/ml) was added to same test tubes.
3. The final concentration to each tube was (170/8, 150/4, 130/2, 110/1, 90/0.5 and 70/0.25 µg/ml)
4. 1ml of gram negative bacterial inoculum after turbidity checker was added to all tubes with control tube (contain bacterial inoculum with sugar) .
5. All tubes were incubated at 37°C for 24 hrs. with dark conditions.

2.8.1.2.2. Combination biological SNPs with Imipenem:

1. 0.5 ml of Imipenem concentration (8, 4, 2, 1 and 0.5 µg/ml) was added to test tubes.
2. 0.5ml of biological SNPs concentration (85, 65, 45, 25 and 5µg/ml) was added to same test tubes.
3. The final concentration to each tube were (85/8, 65/4, 45/2, 25/1 and 5/0.5 µg/ml)
4. 1ml of gram negative bacterial inoculum after turbidity checker were added to all tubes with control tube (contain bacterial inoculum with sugar) .
5. All tubes were incubated at 37°C for 24 hrs. with dark conditions.

2.8.1.2.3. Combination chemical SNPs with Azithromycin:

1. 0.5 ml of Azithromycin concentration (512, 256, 128, 64, 32, 16 and 8 µg/ml) was added to test tubes.
2. 0.5ml of chemical SNPs concentration (170, 150, 130, 110 90, 70 and 50µg/ml) was added to same test tubes.

3. The final concentration to each tube were (170/512, 150/256, 130/128, 110/64, 90/32, 70/16 and 50/8 µg/ml)
4. 1ml of *Staph*. *lentus* inoculum after turbidity checker was added to all tubes with control tube (contain bacterial inoculum with sugar).
5. All tubes were incubated at 37°C for 24 hrs. with dark conditions.

2.8.1.2.4. Combination biological SNPs with Azithromycin:

1. 0.5 ml of Azithromycin concentration (512, 256, 128, 64 and 32 µg/ml) was added to test tubes.
2. 0.5ml of biological SNPs concentration (85, 65, 45, 25 and 5µg/ml) was added to same test tubes.
3. The final concentration to each tube were (85/512, 65/256, 45/128, 25/64 and 5/32 µg/ml)
4. 1ml of *Staph*. *lentus* inoculum after turbidity checker was added to all tubes with control tube (contain bacterial inoculum with sugar).
5. All tubes were incubated at 37°C for 24 hrs. with dark conditions.

MIC is a lowest silver nanoparticles concentration in combination with lowest antibiotics concentration that inhibit completely the bacterial growth and can detected by unaided eye. The turbidity of bacterial growth in tubes containing serial dilution of SNPs and antibiotics combination compared with control tube (without SNPs and antibiotic) and determine the concentration of tube before first one with turbidity to be the Minimum inhibitory concentration. All combination tubes to all methods above were evaluated the gene expression to biofilm in tested bacteria by real time PCR.

2.9. Real time PCR:

2.9.1. Quantitative Reverse Transcriptase Real-Time PCR:

Quantitative Reverse Transcriptase Real-Time PCR technique was performed for measurement of relative quantification (gene expression analysis) for Biofilm genes in experimental bacteria.

Table (2-22) and (2-23) shows the treatments to the biofilm forming bacteria (*S. fonticola*, *Pantoea* sp., *S. lentus*) were used in Real-time PCR experiment:

Table (2-22): Treatment number with MIC solutions of SNPs and antibiotics:

Treatment no.	Name
T1	MIC of chemosynthesis silver nanoparticles
T2	MIC of Biosynthesis silver nanoparticles
T3	MIC of Antibiotics Imipenem or azithromycin
C (control)	Bacterial broth supplemented with sugar

Table (2-23): Treatment number with combination of SNPs with selected antibiotics:

Treatment no.	Chemical SNPs with		Biological SNPs with	
	Imipenem	azithromycin	Imipenem	azithromycin
T1	170/8	170/512	85/8	85/512
T2	150/4	150/256	65/4,	65/256
T3	130/2	130/128	45/2,	45/128
T4	110/1	110/64	25/1	25/64
T5	90/0.5	90/32	5/0.5	5/32
T6	70/0.25	70/16		
T7	50/0.125	50/8		
C (control)	Bacterial broth supplemented with sugar			

2.9.2.Total RNA extraction:

Extraction of total RNA from treatment and control groups of (*S. fonticola*, *Pantoea* sp. and *S. lentus*) were done by using (TRIzol® reagent kit) according to instruction of manufactured company as steps below:

1. Overnight bacterial broth at 1ml were put in eppendrof tube to centrifugation at 1200 rpm, 4C° for 1min. to collect the bacterial cell pellets.
2. 1ml of TRIzol® reagent was added to bacterial cell pellets and vigorously shaken for 1min. then added 200µl of chloroform to each tube and shaken for 15 sec.
3. The mixture was incubated in ice to 5 min. then centrifuged at 12000 rpm, 4C°, for 15 min.to obtain the supernatant.
4. Supernatant was transferred into a new eppendroff tube, and added 500µl isopropanol on it, then mixed by inverting the tube 4-5 times and incubated at 4C° for 10 min. Then, centrifuged at 12,000 rpm , 4C° for 10 min.
5. The supernatant was discarded and added 1ml of 80% Ethanol and mixed by vortex again. Then, centrifuged at 12000 rpm, 4C° for 5 min.
6. The product supernatant was discarded and the RNA pellet was left to dry on air.
7. 50µl of DEPC water was added to RNA pellet to dissolved it, then RNA sample was kept at -20 for further use.

2.9.3.Nanodrop to RNA yield and quality assessment:

The RNA yield and quality assessed and measurement by Nanodrop spectrophotometer. There are 3 quality controls were performed on RNA sample in same nanodrop apparatus. The first one being the quantity of RNA (ng/µL) was determined and the purity of

RNA sample was evaluated by reading the absorbance in spectrophotometer at 260 nm and 280 nm.

2.9.4.DNase I treatment:

The extracted RNA was treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA, by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow:

Table (2-24): Components Reaction and their volumes used for DNase I treatment

Mix	Volume
Total RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total	20ul

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop solution was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

2.9.5.cDNA synthesis:

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as following table:

Table (2-25): Reverse transcriptase master mix with their volumes for cDNA synthesis

RT master mix	Volume
Total RNA 100ng/ul	10ul
Random Hexamer primer	1ul
DEPC water	9ul
Total	20ul

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then dissolved completely by vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Table (2-26): Thermocycler condition for cDNA synthesis

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.10. Quantitative Real-Time PCR (qPCR):

Relative quantification by Real-Time PCR was performed for determination of gene expression (mRNA transcript levels) of Biofilm genes for tested bacteria that normalization by housekeeping gene *rpo*B gene. This method was carried out by AccuPower Green star qPCR premix for biofilm gene (Table 2-27) and for housekeeping gene (Table 2-28):

Table (2-27): qPCR master mix protocol for biofilm gene.

qPCR master mix		volume
cDNA template (20ng) for Treatment or control isolates groups		2.5µL
Primers (Biofilm gene) (10pmol)	Forward primer	1 µL
	Reverse primer	1 µL
DEPC water		13 µL
Total		20 µL

Table (2-28): qPCR master mix prtocol for house keeping gene (*rpoB*).

qPCR master mix		volume
cDNA template (20ng) for Treatment or control samples		5µL
Primers (rpoB gene) (10pmol)	Forward primer	1 µL
	Reverse primer	1 µL
DEPC water		13 µL
Total		20 µL

After that, these qPCR master mix component that mentioned above AccuPower Green star qPCR premixed standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Exicycler Real-Time PCR system:

After that, the qPCR plate was loaded and the following thermocycler protocol (Table 2-29):

Table (2-29): the thermocycler real time PCR protocol:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	50 °C	1 hour	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

2.10.1. Data analysis of qRT-PCR

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene) that described by (Livak and Schmittgen, 2001). as following equation:

$$\textbf{Ratio (reference/target)} = 2^{\textbf{CT(reference)} - \textbf{CT(target)}}$$

2.11. Statistical analysis:

Each experiment was repeated three times and to validate the reproducibility of the experiments. Statistical analysis was done by using t test and one way ANOVA at *P* value 0.05 by SPSS Statistics 24.0 software.

Chapter Three

Results and Discussion

3. Results and discussion:

3.1. Biofilm formation:

Among 65 urine samples there were 58 (89.2%) gave a positive bacterial growth, 28 (48%) bacteria had an ability to form biofilm (Fig. 3-1).

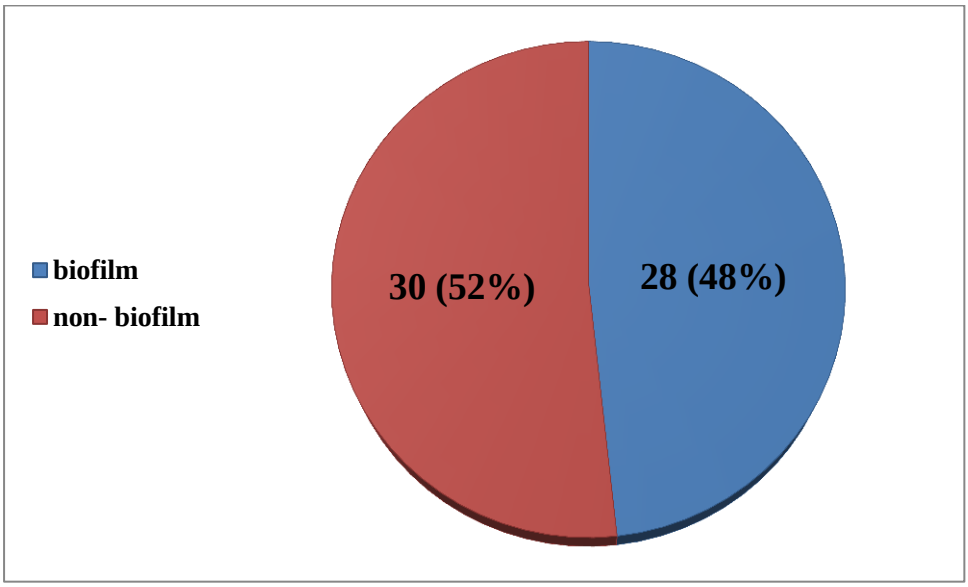


Figure (3-1): Number and percentage of bacterial biofilm formation among catheterized patients.

The highly occurrence of biofilm in urine samples may attribute to virulence determinants for bacterial persistent in urinary tract (Costerton *et al*., 1999). These results corresponded with (Salih and AL-Ani, 2013) whom found 81.4% were positive bacterial growth isolated from catheter urine specimens, 80.3% of isolated bacteria were biofilm producer higher than the present study. Authors found that biofilm formation was 58.66% and no biofilm formation in 41.35% from urine samples (Kaur and Sanjivani, 2015). Several studies have found that the majority of biofilm producing bacteria was from urinary catheter (Donlan, 2001, Hassan *et al*., 2011). The bacterial adhesion on the catheter was depended on multi-factors such as charge of catheter surface, hydrophility and

hydrophobicity of catheter, period of indwelling, pH of urine and bacterial cell and on own bacterial adhesion genes (Hola and Ruzicka, 2011).

Urinary catheters prompt to UTI by damaging protective barriers and providing a seed for infection which induces fibrinogen release as a result of inflammatory response; to accumulates in the bladder and further on the catheter and act as a substrate to form biofilm (Delcaru *et al*., 2016).

Bacterial isolates that produced biofilm on Congo red media (Fig. 3-2) where the black color indicated the ability of Congo red dye to stain the polysaccharide matrix which formed during the biofilm forming process (Bose *et al*., 2009).

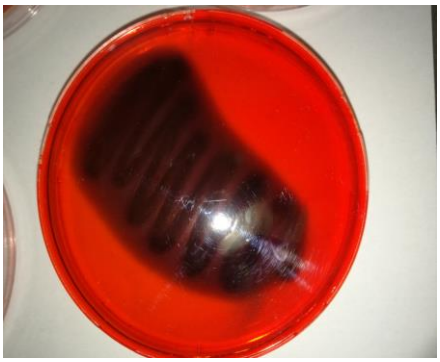


Figure (3-2): Congo red agar indicating the biofilm formation.

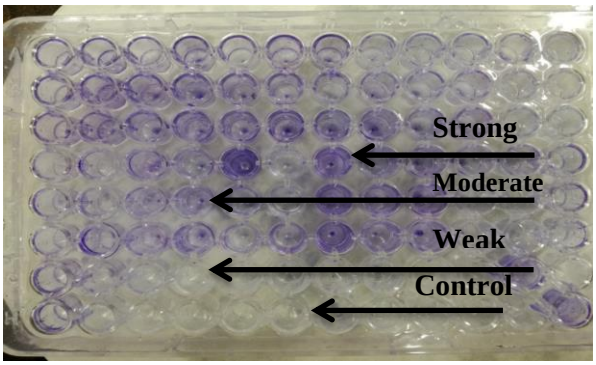


Figure (3-3): TCP method indicating the biofilm degree of bacteria.

In TCP method, the bacterial isolates showed a strong, moderate and weak or non-biofilm production (Fig. 3-3). The Results were interpreted according to (Christensen *et al*., 1985).

Among bacterial species, the biofilm formation ability was different from species to other. (Hola and Ruzicka, 2011) pointed that some bacterial species with high ratio of biofilm formation whereas other with low ability and that differences were significantly ($p< 0.05$). The results revealed that *Proteus mirabilis, Pantoea* sp., *E. coli and Yersinia*

enterocolitica at strong biofilm producers (Table 3-1), with a significant difference than other isolates as (Appendix 2). It is consistent with other study which has found that *Enterococcus faecalis, Proteus mirabilis* and *Staphylococcus aureus* were highly strong biofilm (Hola and Ruzicka, 2011).

Table (3-1):Biofilm Production Capacity (OD_{630} nm) of bacterial isolates.

Biofilm bacteria	Biofilm degree		
	Strong (%)	Moderate (%)	Week(%)
S. lentus	1(33)	2(66)	0
P. mirabilis	2(40)	3(60)	0
K. pneumonia	1(25)	3(75)	0
S. fonticola	2(66)	1(33)	0
Pantoea sp.	4(100)	0	0
E. coli	2(100)	0	0
P. oryzihabitance	1(33)	2(66)	0
E. aeruginosa	0	2(100)	0
E. cloacae	1(100)	0	0
Y. enterocolitica	1(100)	0	0

1. Duration of catheter time with biofilm:

The current results were revealed that the duration of catheter time at one day were more predispose to biofilm formation as (Fig. 3-4), and that may explained by highly virulence of causative bacteria and ability to biofilm formed, and that result was corresponded with other studies were pointed that biofilm may formed immediately after insertion of catheter, that related to microorganisms ability to attach in short time to catheter surface (Anghel *et al*., 2013). However other studies showed the remaining urinary catheters for long time in urinary tract were more likely to predispose biofilm formation, when the catheter remain at least 7 days at 50% probability become infected (Stickler, 1996).

The challenge of biofilm infection depend on duration of catheterization and catheter management (Hola and Ruzicka, 2011). In

general, if catheterization at short time with sterile techniques is used in catheter use, removal and re-insertion, that reduce incidence of a catheter associated urinary tract infection (Topal *et al*., 2005).

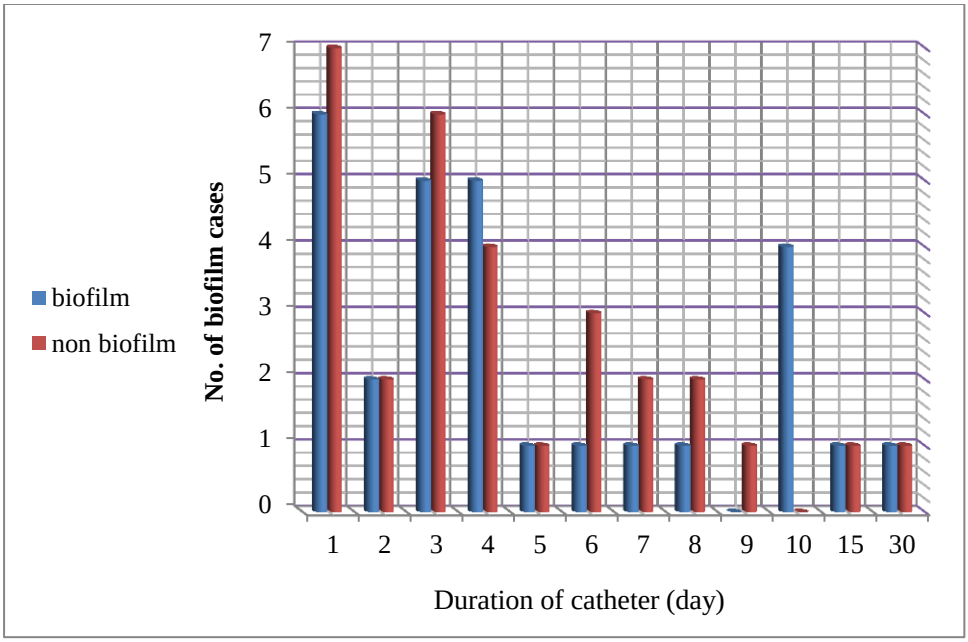


Figure (3-4): Number of biofilm and non- biofilm formation according to duration of catheter (day)

2. Age of patients:

The present study revealed that elderly patients more susceptible to biofilm formation (Fig. 3-5).The results showed that patients at 50-60 years old more liable to biofilm formation in urinary catheter than other age groups may be that related to that patients in at these age group haunted to the hospital frequently in addition to decline their immune state.

The urinary catheter is the most common catheter used for long term hospitalized patients and the elderly in long-term community health care with bladder dysfunctions and incontinence (Getliffe and Newton, 2006).

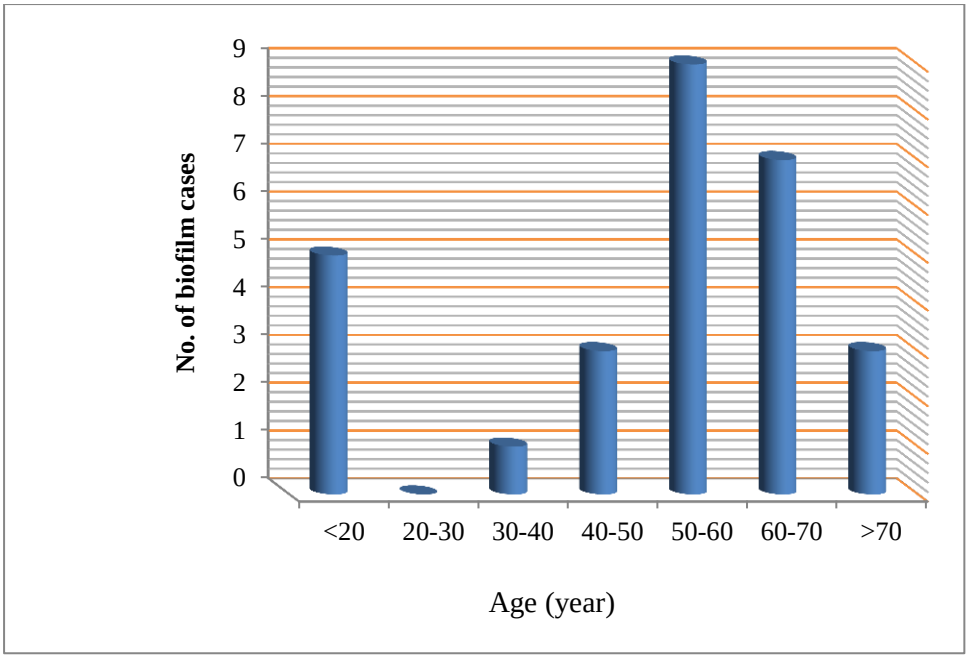


Figure (3-5): Number of biofilm cases according to patients age groups.

3. Sex of patients:

Among 28 catheterized patients have biofilm formation, there were 13 (46.4%) male and 15 (53.5%) female. The female was more than male because they have more predisposing factors to infection than the male. UTI is higher in women than in men, which is likely the result of several clinical factors including anatomic differences, hormonal effects, and behavior patterns (Hindi *et al*., 2013) .

Female are more prone to UTIs than male due to the proximity of the urethra, vagina, and rectum. Host factors such as changes in normal vaginal microbiota may also increase the risk of UTI in females which may lead to recurrent infection. Recurrent UTI more predispose to biofilm formation because the uropathogenic bacteria possesses many redundant virulence factors that allow bacteria to resist and overcome various host defense mechanisms, namely, type 1 fimbriae and pili involved in adherence to host cells (Delcaru *et al*., 2016).

4. Clinical cases associated with biofilm formation:

The data was documented from medical history sheet of patients and associated the clinical case with biofilm formation bacterial isolates were listed in (Table 3-2). The table shows that most patients in this study admitted to the hospital without UTI disorder, might have other clinical cases.

Other researches revealed that biofilm forming ***Klebsiella pneumonia*** associated with kidney disease or bone fracture (Hassan *et al*., 2011) same as present results.

Table 3-2: Clinical cases associated with biofilm forming bacterial isolates:

Bacterial isolates	No. of cases	Clinical cases
Proteus mirabilis	**5**	Intestinal obstruction
		Bladder surgery
		Renal failure
		Renal failure
		Leg fracture
Klebsiella pneumonia	**4**	Prostoctomy
		Renal stone
		Leg fracture
		CVA
Serratia fonticola	**3**	Ovary cancer
		Tuberculosis
		Cancer
***Pantoea* Sp.**	**4**	CVA
		CVA
		Renal stone
		Spondylolishesis
Pseudomonas oryzihabitans	**3**	Prostoctomy
		CVA

		Leg fracture
E. coli	**2**	Paralysis
		CVA
Enterobacter aerogenes	**2**	Angina
		Coma
Enterobacter cloaca	**1**	Colon cancer
Yersinia enterocolitteca	**1**	Uteroctomy
S. lentus	**3**	Cesarean section
		CVA
		Arm fracture

CVA: Cerebrovascular accident

Serratia sp. were frequently colonized or infected hospitalized patients. It was regarded as a significant opportunistic pathogen causing infection in immunodeficient patients (Tariq and Prabakaran, 2010). So in present study ***Serratia*** associated with immuncompromized cases such as cancer as shown in table above. Bacteria adherence to uroepithelium may invade the renal tissue to cause chronic prostatitis and pyelonephritis (Delcaru *et al*., 2016).

According to the National Institutes of Health (NIH), urology is one of the main areas of concern where biofilm can become a serious problem and biofilm are found in the urothelium, prostate stones, and implanted foreign bodies (Tenke *et al*., 2006).

3.2. Bacterial etiology of biofilm:

The current study found that gram negative bacteria were forming biofilm more than gram positive bacteria (Fig. 3-6). The microorganisms that present in priurethral skin and colonize on it have ability to migrate to urinary bladder as a result of biofilm formation between urethra epithelial surface and the catheter or by contamination of urine drainage

bag with bacteria allow it to access the urinary bladder through the lumen of catheters tube (Stickler, 2008).

Gram positive and negative bacteria have important role in UTIs, but a gram negative bacteria was a main nosocomial pathogens cause UTI especially in catheter associated UTI as prevalent pathogens (Hatt, 2008) as well as biofilm formation.

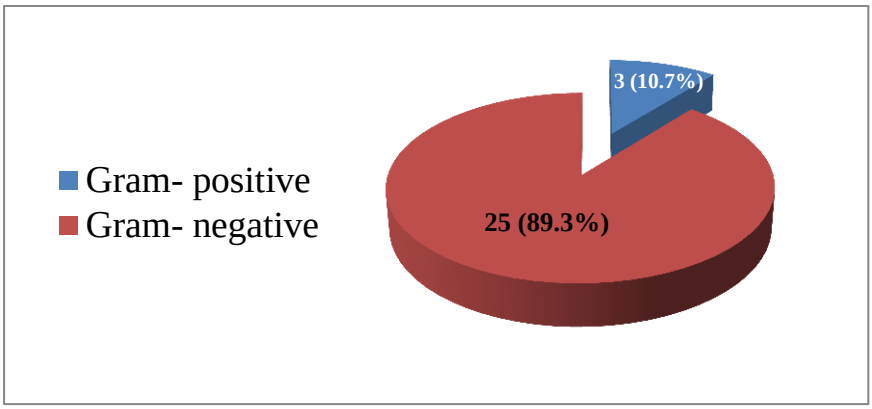


Figure 3-6: Number and percentage of biofilm formation according to bacterial groups.

The biofilm bacterial isolates distributed as gram positive (10.7%) represented by (*Staphylococcus lentus*) only and gram negative (89.3%) (Fig. 3-7).

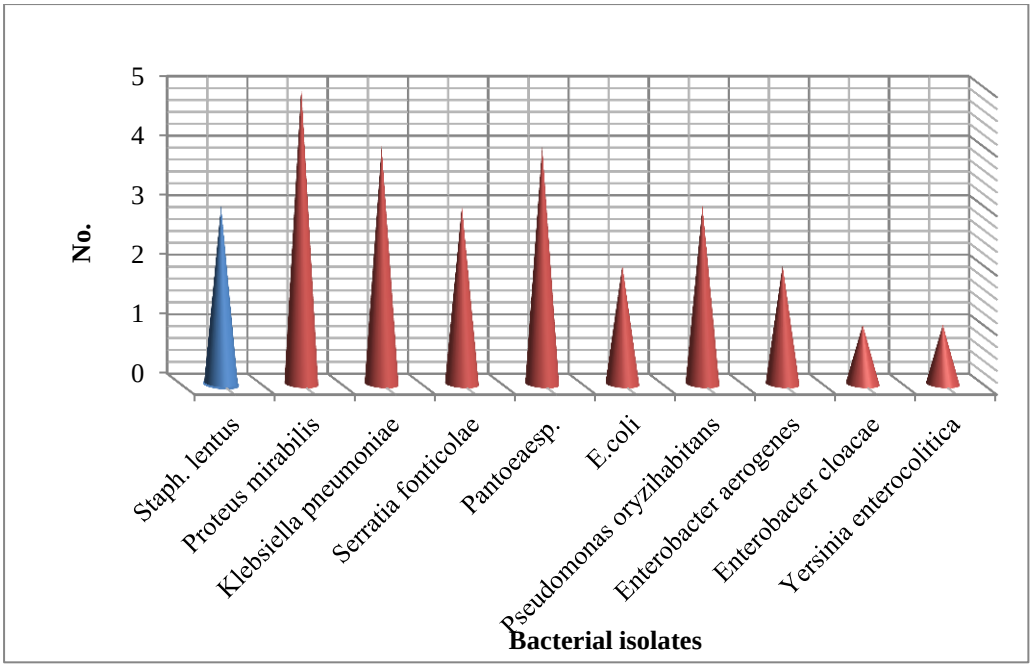


Figure (3-7): Number of biofilm bacterial isolates in urinary catheter.

The results revealed that most bacterial isolates biofilm formation were belong to Enterobactericeae family. In current study, biofilm formation were associated with urinary catheter infections are mostly caused by Enterobactericeae microflora, gram negative rods (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* sp., *Pseudomonas aeruginosa*, *Proteus mirabilis* etc.) (Tenke *et al*., 2006), who thought that tis group of bacteria were derived largely from the patient's gut microbiota . The most common routes of urinary tract infection mainly involve fecal flora ascending to the bladder and kidneys via the urethra. The bacteria that colonize the distal urethra attach to the external surface or lumen of the catheter, as it is inserted into the bladder (Hooton, 2000, Jacobsen et al., 2008). Another common route of infection is incomplete voiding of the bladder, whereby bacteria from the urinary meatus migrate to the bladder and proliferate using the urine as a nutrient source. They can then colonize the catheter (Hashmi *et al*., 2003).

Moreover, the results showed that ***Proteus mirabilis*** was predominant than other bacteria in 5 isolates (17.8%). *Proteus* sp. as uropathogene was opportunistic bacteria in susceptible patients such as those with long- term catheterization (Mobley and Warren, 1987), It was perhaps ascend to patient's urinary bladder through 1-3 days (Pearce et al., 1995); and that may enhance by swarming motility of *Proteus* sp. to facility the movement (Donlan, 2001). Urease produced by *Proteus* sp. breaking down urea and generates alkaline conditions which lead to biofilm formation on catheter surface (Holling, 2014). Urease enzyme hydrolyze urea to ammonia, elevation ammonia concentration lead to raise urinary pH which lead to urinary salt precipitation to form kidney and bladder stones (Tabibian *et al*., 2008). Urinary salts calcium and magnesium ammonium phosphate when precipitation, it incorporated with microbial polysaccharide to form crystalline biofilm on catheter surface (Flores-Mireles *et al*., 2014). Bacteria capable to produce urease are (*Pseudomonas* sp., *Proteus* sp.. *Klebsiella pneumonia*, *Morgenella* sp. *S. aureus* and coagulase negative staphylococci.) (Stickler, 2008).

The second predominant bacterial isolates were *Klebsiella pneumonia* and *Pantoea* sp. at 4 isolates (14.2%) to each one. *Pantoea* sp. was belong to Enterobactericeae it was previously named *Enterobacter* and *Erwinia* (Kazancioglu *et al*., 2014). It is opportunistic pathogen can cause diseases to hospitalized patients especially those with immune compromised infection when exposure to contaminated equipment or fluids with these bacteria (Dutkiewicz *et al*., 2016). Recently *Pantoea* sp. recovered from humans which consider an opportunistic pathogen associated with contaminated catheters (Delétoile *et al*., 2009, AbdAlhussen and Darweesh, 2016). Some species drive different gene expression by quorum sensing ability which hence controlling

physiological activities (Tan *et al*., 2014) involving biofilm formation. Many studies were isolated *Pantoea* sp. from UTI patients (Cruz *et al*., 2007, Büyükcam *et al*., 2017) were isolated *Pantoea* from different specimens of human body, and was found 10% and 21.4% isolates from UTI patients respectively. *Klebsiella pneumonia* and *Pseudomonas aeruginosa* has been isolated also from biofilms on Urinary catheter (Donlan, 2002).

The main bacterial virulence factors involve in biofilm formation is urease production and different types of motility. Most Enterobactericeae members were urease positive, the most significantly, *Proteus* sp., *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Hola and Ruzicka, 2011), also the type of motility can facilitate the movement of bacteria and attach to catheter surface to form biofilm.

Serratia is considered as opportunistic human pathogen, it was achieved a great number cases of nosocomial infections with seriously problem of multi- drug resistance (MDR) (Mun *et al*., 2013). A large number of *Serratia* sp. are found to be isolated from urinary tract clinical samples (Henriques *et al*., 2013, Ee *et al*., 2014). Manikandan and coworker reported that ***Serratia*** sp. were the least dominant uropathogen causing UTI (Manikandan *et al*., 2011), although the ability to form biofilm may contribute to its pathogenicity to show them as clinical importance (Hirata et al., 2006). It have flagella-mediated swimming and swarming motility which associated biofilm formation and that facilitate the contact between bacterial cells and solid materials (Eberl *et al*., 1999), that may explain the ability of ***Serratia*** sp. to form biofilm phenomenon on urinary catheter. Biofilm production has been reported for several

Serratia sp. (Liu *et al*., 2011). *Serratia fonticola* represent in (10.7%) at 3 isolates in current study.

While the gram positive biofilm bacteria which represent in *Staphylococcus lentus* in 3 isolates (10.7%). *Staphylococcus lentus* (*S. lentus*) is recognized as opportunistic pathogens and rarely causing infections to human (Stepanovic *et al*., 2003) but recently caused nosocomial and community infections (Koksal *et al*., 2009). *S. lentus* is a gram positive, coagulase-negative staphylococci (CoNS), it is mostly infectious to animal species and may colonize humans, which has been reported to have been isolated from various human clinical specimens (Nagase *et al*., 2002). CoNS are considered to be an uncertain pathogenic in the urinary tract (Aspevall *et al*., 2001). However, studies suggested that significance of some CoNS as uropathogens may have been undervalued (Guirguitzova *et al*., 2002), *Staphylococcus* sp. is the main cause of (CAUTI) (Gad *et al*., 2009). Several investigators have reported the isolation of *S. lentus* from urine (Guirguitzova *et al*., 2002). Bose *et al*. found that coagulase negative Staphylococci was generally the main cause of biofilm forming on indwelling medical devices (Bose *et al*., 2009). This capability is due to that coagulase negative Staphylococci is capable to produce urease enzyme (Stickler, 2008). Many local studies showed a high percentage of biofilm formation among *Staphylococcus* sp. especially CoNS with the ability of multidrug resistance (MDR) (Mohammed *et al*., 2015, Bakir and Ali, 2016), but there have been no studies about *S. lentus* exclusively.

3.3. Molecular assay:

The molecular assay was done for (*Pantoea* sp., *Serratia fonticola* and *Staphylococcus lentus*) which they isolated at the first time locally as biofilm forming bacteria from urinary catheterized patients.

1. *Staphylococcus lentus:*

All 3 isolates of *S. lentus* were tested for the presence of *icaA* gene (intracellular adhesion gene). The results showed that all isolates were positive for this gene (Fig. 3-8) (Amplicon size 77 bp). These findings were indicated by Gad *et al*. that this gene was present in other *Staphylococcus* sp. (*S. aureus*, *S. epidermidis*) (Gad *et al*., 2009). Many researches indicated that *ica* genes had an important role as the virulence factor of staphylococcal infections associated with urinary catheter (Arciola *et al*., 2001, Gad *et al*., 2009). The *icaA* gene among *ica* genes has a significant role in biofilm formation in *Staphylococcus* sp. (Yazdani *et al*., 2006), encoding N-acetylglucosaminyltransferase which is involved in the synthesis of polysaccharide intercellular adhesion (PIA) and β-1-6-linked poly-Nacetylglucosamine polymer (PNAG) that lead to expressing adhesion and capsular polysaccharide phenotypic character (Arciola *et al*., 2001, Heilmann, 2003). That may explain the *Staphylococcus* sp. to colonize artificial materials and assist adsorption on biomaterial and solid surface (Montanaro *et al*., 1998). Other researches showed no biofilm formation by *Staphylococcus* sp. when was negative for *icaA* gene, which might be due to the lack of the entire *ica ADBC* operon (Montanaro *et al*., 1998, Arciola *et al*., 2001).

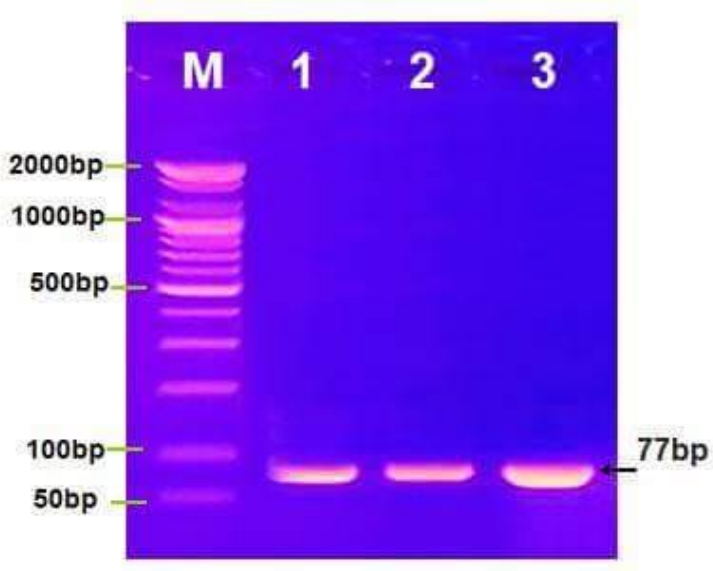


Figure (3-8): Agarose gel electrophoresis of PCR assay showed the positive results of biofilm formation of *icaA* gene in *Staphylococcus* sp. positive isolates. Lane (M): DNA marker (50-2000 bp); Lanes (1-3): Positive *icaA* at 77 bp PCR product size.

2. *Serratia fonticola:*

The amplification of *smaI* gene (quorum sensing gene) in *S. fonticola* isolates shows in (Fig. 3-9). The results revealed that bacterial isolates were positive to *smaI* gene (Amplicon size 73bp).

Biofilm formation and swarming motility of *Serratia* sp. are regulated by quorum sensing (Horinouchi, 2010), *smaI* is a quorum sensing-regulated gene that is involved in biofilm development (Van Houdt et al., 2007), which regulate activity of hemolysis, swarming motility and biofilm formation (Coulthurst *et al*., 2006, Mahlen, 2011). Quorum sensing (QS) It is small chemical molecules called autoinducer have main role in attachment and induce bacterial cell to aggregate with each other to form biofilm (Fuqua et al., 2001, Miller and Bassler, 2001).

Coulthurst *et al*. shows that biofilm formation in ***Serratia*** sp. was dependent on *smaI* gene, detected by tissue culture plate (TCP) as biofilm formation indication (Coulthurst *et al*., 2006). The biofilm form under control of AHL-dependent quorum sensing in this bacteria (Van Houdt *et*

al., 2007). In 2014 provided evidence for the first time the presence of quorum sensing activity in ***Serratia fonticola*** by confirmed a three short chain AHLs(Ee *et al*., 2014).

Serratia sp. were expressed QS system (*SmaI*) by utilizes C4-homoserine lactone (C4-HSL) as signal molecules and directs the production of various virulence factors like prodigiosin, protease, heamolysin production and most significantly biofilm formation (Rice *et al*., 2005). Quorum sensing system (*smaI*) appears to play a role in regulating biofilm production for *Serratia* sp., as described above.

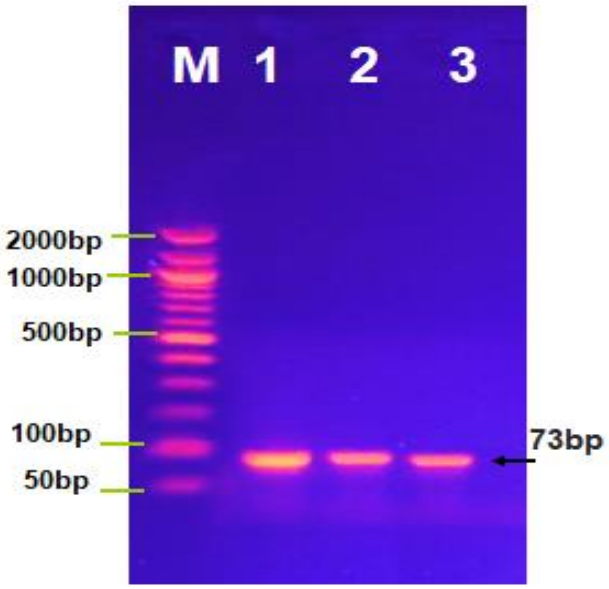


Figure (3-9): Agarose gel electrophoresis of PCR assay show the some positive results of biofilm formation *smaI* gene in *Serratia* sp. positive isolates. Where, Lane (M) DNA marker (50-2000bp), Lane (1-3) positive *smaI* at 73 bp PCR product size.

3. *Pantoea* sp.:

PCR analysis of *Pantoea* sp. revealed that the 4 *Pantoea* isolates have *esal* gene (Fig. 3-10) (Amplicon size 127 bp) which mean have ability to

synthesize quorum sensing signaling molecules (N-acylhomoserine lactones (AHLs)) and biofilm formation, likely to (Morohoshi *et al*., 2011, Von Bodman *et al*., 1998), which pointed that *Pantoea* members have been reported to synthesis QS which regulate many phenotypes, such as production of virulence factor, aggregation of cells and biofilm formation (Morohoshi *et al*., 2007).

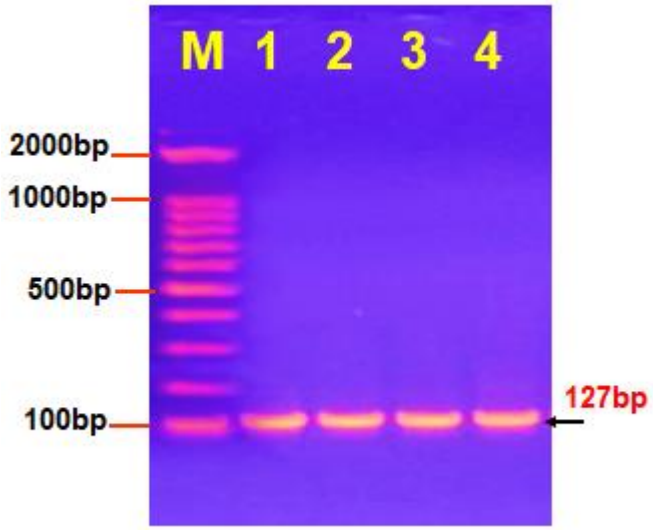


Figure (3-10): Agarose gel electrophoresis of PCR assay show the some positive results of biofilm formation *esaI* gene in *Pantoea* sp. positive isolates. Where, Lane (M) DNA marker (100-2000bp), Lane (1-4) positive *esaI* at 127 bp PCR product size.

Pantoea sp. shows QS activity via increase cell density- dependent Exoplysaccharide synthesis (Yunos *et al*., 2014, Tan *et al*., 2014). Exoplysaccharide (EPS) is a major component of biofilm bacterial matrix and a powerful virulence factor which protect the bacterial cell from antibiotics action and host immune defense (Minogue *et al*., 2005). *esal* quorum sensing gene was governed synthesis the exopolysaccharide (EPS) in *Pantoea* sp. to appropriate bacterial adhesion and biofilm formation (Koutsoudis et al., 2006). *esal* gene consider a typical N-acyl-L

homoserine lactones (AHLs) synthase which catalyzes synthesis of N-3-oxo-hexanoyl homoserine lactone (Watson *et al*., 2002).

Other studies suggest that *esal* gene mutant happen lead to lack adhesion ability and biofilm formation by inhibit AHL synthesis (Koutsoudis *et al*., 2006). In addition to degradation of QS by chemical or biological inhibitors may inhibit biofilm formation (Rasmussen *et al*., 2005).

Most the studies cited about *Pantoea* sp. *esal* gene study were that associated with plant diseases only (Minogue *et al*., 2002, Koutsoudis *et al*., 2006, Mohamad *et al*., 2015), no study about *Pantoea esal* gene in human diseases.

3.4. Antimicrobial susceptibility testing:

The current study focuses on determining and evaluating antibiotic resistance in biofilm forming urobacteria (*Staphylococcus lentus, Serratia fonticola* and *Pantoea* sp.) by three methods, disc diffusion; MIC in VITEK2 AST and manual MIC for imipenem and azithromycin only.

3.4.1. Disc diffusion and VITEK2 AST method:

These methods were done to tested biofilm forming bacteria (*Staphylococcus lentus, Serratia fonticola* and *Pantoea* sp.).

1. *S. lentus:*

S. lentus displayed resistance to multi- antibiotics and was tested in two methods: disc diffusion (Fig. 3-11); MIC in VITEK2 AST (Table 3-4). In disc diffusion method, the results displayed that 3 isolates of *S. lentus* were resistant to most antibiotics tested in the experiment. *S. lentus* isolates revealed a resistance rate of 100% to each penicillin, ciprofloxacin, carbencillin, gentamycin and tobramycin except azithromycin which was effected on *S. lentus* at a resistance rate of 0%

which the mean inhibition zone of 18 mm in contrast to non- biofilm control (CoNS) which also revealed antibiotic resistance but with high sensitivity to AZM at 25mm and intermediate to CIP at 16mm inhibition zone (Fig. 3-11) with a significant difference between *S. lentus* isolates and control at P-value≤ 0.05 (Table 3-3). Many studies have shown that biofilm forming bacteria are more resistant to antibiotics than planktonic bacteria (O'gara and Humphreys, 2001, Smith and Hunter, 2008, Corona and Martinez, 2013). Azithromycin is a member of macrolide group, although there are only limited data available about anti-gram positive biofilm properties of macrolides (Parra-Ruiz *et al*., 2012), but today macrolide antibiotics and especially azithromycin have recently been found to have anti-biofilm activity as shown by restrict bacterial quorum sensing, and inhibition of polysaccharide synthesis to prevent biofilm formation and its relation to the *ica* genes of *Staphylococcus* sp. (Wang *et al*., 2010).

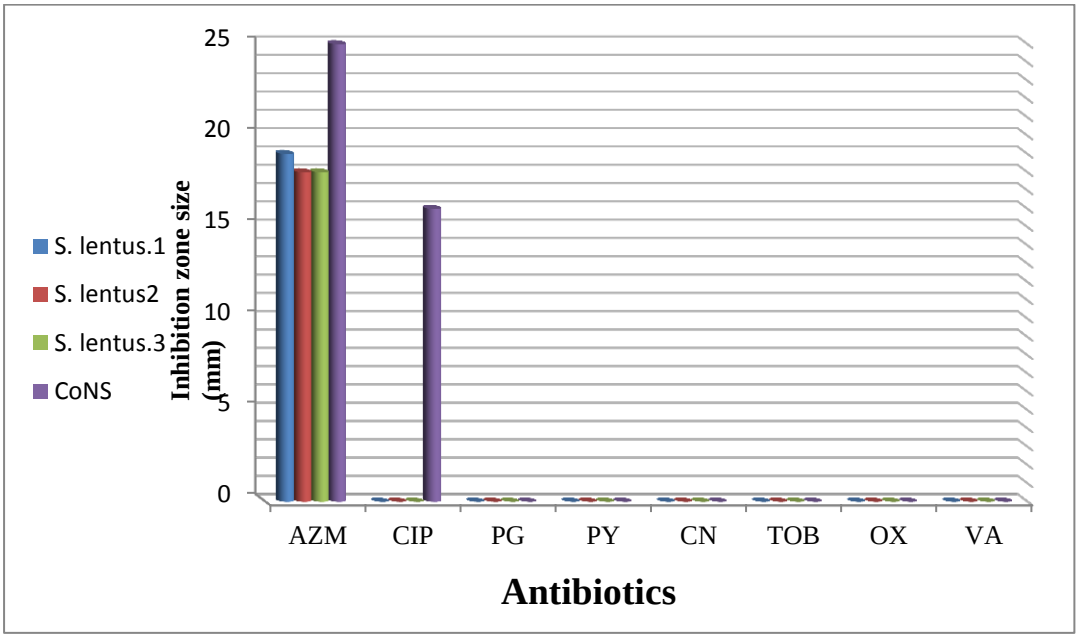


Figure (3-11): Zone of inhibition (mm) of different antibiotics in disc diffusion method against *S. lentus* and CoNS as control (AZM= azithromycin; CIP= ciprofloxacin; PG= penicillin G; PY= Carbencillin; CN= gentamycin; TOB= tobramycin; OX= Oxacillin; VA= Vancomycin.)

Table (3-3): Statistical analysis of susceptibility of *S. lentus* isolates and control to azithromycin according to inhibition zone.

Bacteria	Inhibition zone (mm)
S. lentus 1	18.00 ± 0.527 A
S. lentus 2	18.00 ± 0.020 A
S. lentus 3	18.00±0.333 A
CoNS	23.00±2.888 B

Note: values represent mean ±S.E

Different capital letters mean significant differences (P≤0.05) between different inhibition zones.

In VITEK AST results (Table 3-4), *S. lentus* was resistant to all antibiotics. According to CLSI guidelines, the cefoxitin screen which was tested for *Staphylococcus* isolates predicted results for *mecA*-mediated oxacillin resistance (CLSI, 2017). The *mecA* gene in *Staphylococcus* sp. is responsible for antibiotic resistance (Al-Azawi, 2013). VITEK AST revealed that *S. lentus* was positive for the tested cefoxitin screen, that is, *S. lentus* isolates were methicillin resistant which was confirmed by oxacillin disc test in disc diffusion test, in addition to its resistance to vancomycin too, to be methicillin resistant coagulase negative staphylococci (MRCoNS) and Vancomycin resistant coagulase staphylococci (VRCoNS). Methicillin resistant staphylococci resisted all β-lactam antibiotics (cephalosporins, penicillins) and aminoglycosides, which has been considered multi-drugs resistance (MDR) (Cihalova *et al*., 2015, CLSI, 2017).

Table (3-4): VITEK2 antibiotic sensitivity test (AST) system results for *S. lentus*:

Antibiotics	MIC	Interp.	Antibiotics	MIC	Interp.
Beta-lactamase	NEG	—	**Erythromycin**	≥ 8	R

Cefoxitin screen	POS	+	**Clindamycin**	≥ 8	R
Benzylepencillin	≥ 0.5	R	**Teicoplanin**	≥ 32	R
Oxacillin	≥ 4	R	**Vancomycin**	≥ 32	R
Gentamicin	≥ 16	R	**Tetracycline**	≥ 16	R
Tobramycin	≥ 16	R	**Nitrofurantain**	256	R
Levofloxacin	≥ 8	R	**Rifampicin**	≥ 32	R
Moxifloxacin	≥ 8	R	**Trimethoprim/Sulfamethoxazole**	≥320	R
Inducible clindamycin resistance	NEG	—			

Note: R=resistant, NEG= negative, POS=positive.

2. *S. fonticola* and *Pantoea* sp.:

S. fonticola and *Pantoea* sp. recovered as multi- drug resistance. Antimicrobial susceptibility testing was doing by two method (disc diffusion and MIC by VITEK AST). In disc diffusion method, all isolates of (3 *Serratia* and 4 *Pantoea* sp.) (Fig. 3-12and 3-13) respectively, were display resistant rate 100% to each (amikacin, tobramycin, carbencillin, clindamycin and penicillin) and *S. fonticola* revealed sensitivity rate at 100% to each (imipenem and azithromycin) while *Pantoea* sp. revealed 100% to imipenem and 50% to azithromycin. On the other hand the control non- biofilm bacteria (*S. marcescens* and *E. coli*) show highly sensitivity to (imipenem, amikacin, azithromycin and tobramycin) at 100% to each one in comparison to *S. fonticola* and *Pantoea* sp. sensitivity results respectively.

Statistical analysis of susceptibility to imipenem revealed there were significant difference among *Pantoea* sp. isolates and between the isolates and control at $p<0.05$ (Appendix 3). While *S. fonticola* show a significant difference between its isolates and bacterial control (Appendix 4) $p<0.05$.

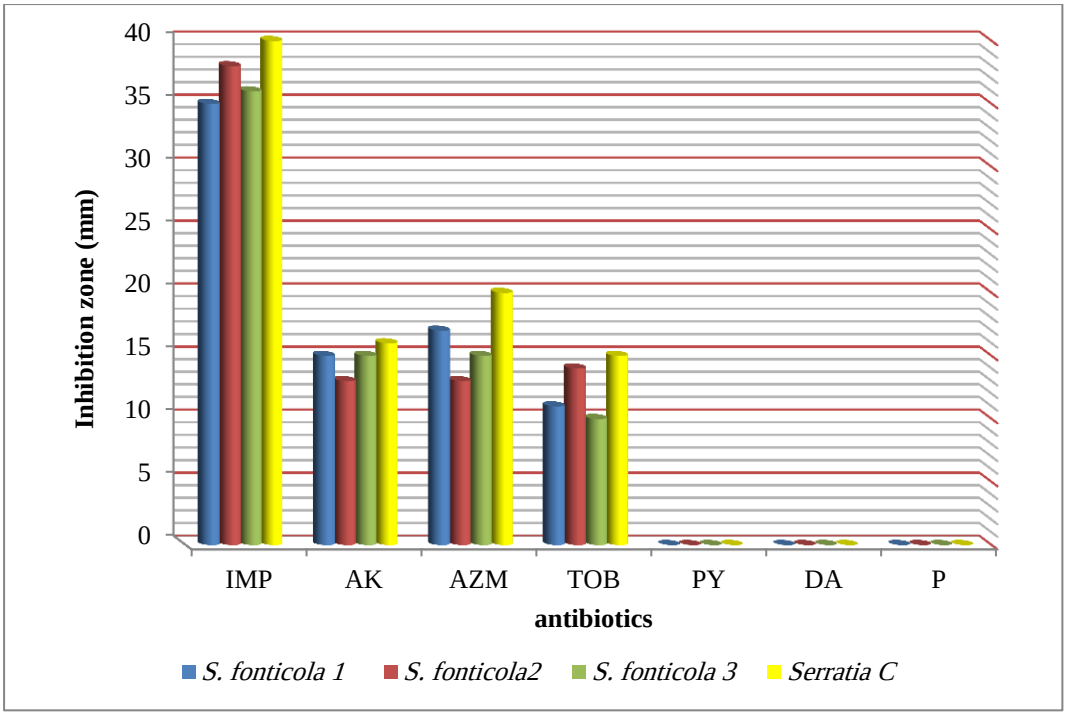


Figure (3-12): Zone of inhibition (mm) of different antibiotics in disc diffusion method against *S. fonticola* and *S. marcescens* as control ((IMP= imipenem; AK= Amikacin; AZM= azithromycin; TOB= tobramycin PY= Carbencillin; DA= clindamycin; P= penicillin.)

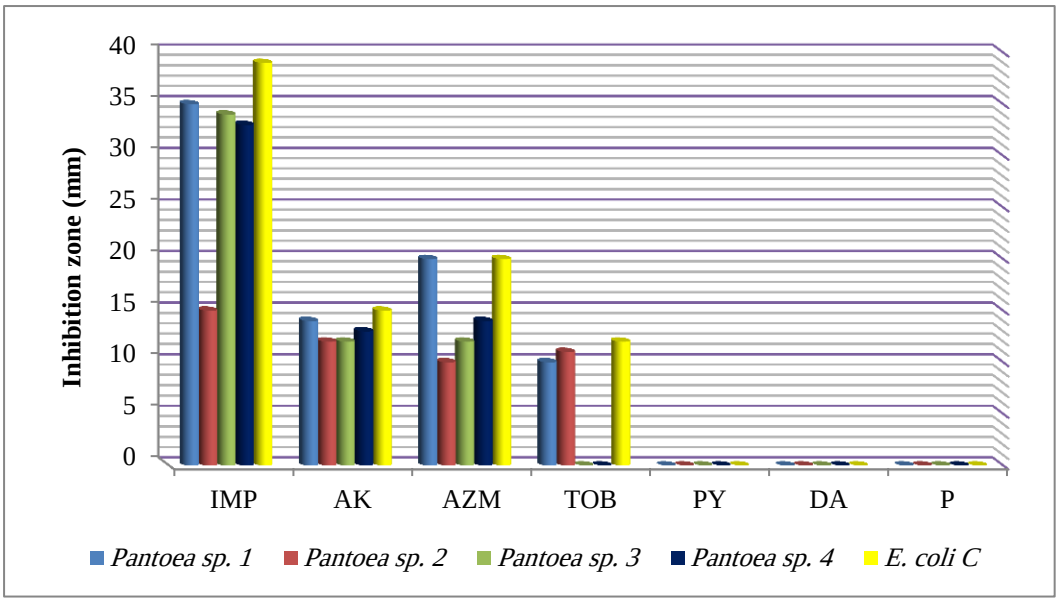


Figure (3-13): Zone of inhibition (mm) of different antibiotics in disc diffusion method against *Pantoea* sp. and *E. coli* as control (IMP= imipenem; AK= Amikacin; AZM= azithromycin; TOB= tobramycin PY= Carbencillin; DA= clindamycin; P= penicillin.).

Some studies were pointed that non biofilm *Pantoea* sp. were uniformly susceptible to most antibiotics tested in their studies (Mardaneh and Dallal, 2013, Kazancioglu *et al*., 2014), but biofilm *Pantoea* sp. were less susceptible to antibiotics at 1000 times than free planktonic bacteria (Donlan, 2000).

Imipenem was the most antibiotic effective on biofilm forming gram negative bacteria at 100% sensitivity. It was used to treat complicated urinary tract infections (CLSI, 2017). Imipenem is a β-lactam antibiotic were resist β-lactamase producer bacteria which remain very stable against penicillinase and cephalosporinase bacteria. it has broad spectrum action on gram positive and gram negative bacteria (CLSI, 2012). It acts as cell wall synthesis inhibitor by binding to penicillin binding proteins (PBPs) to cause loss of cell wall integrity and cell lysis leading to rapid bacterial cell death (Association and Britain, 2015).

Table (3-5): VITEK2 antibiotic sensitivity test (AST) system results for *S. fonticola* and *Pantoea* sp.:

Antibiotics	*S. fonticola*		*Pantoea* sp.	
	MIC	Interp.	MIC	Interp.
Ampicillin/clavulanic	≥ 32	R	≥ 32	R
Cefazolin	≥64	R	≥64	R
Ceftazidime	≥64	R	≥64	R
Ceftriaxone	≥64	R	≥64	R
Cefepime	≥64	R	16	R
Ertapenem	≥0.5	S	≥ 8	R
Imipenem	≥2	I	≥ 16	R
Gentamicin	≥ 16	R	≥ 16	R
Tobramycin	≥ 16	R	≥ 16	R
Ciprofloxacin	≥ 4	R	2	I
Levofloxacin	≥ 8	R	4	I
Nitrofurantain	128	R	128	R
Trimetheprium/sulfamithaxzol	≥ 320	R	≥320	R

R=resistant; I= intermediate

In VITEK AST analysis *S. fonticola* and *Pantoea* sp. revealed resistant rate at 100% to most antibiotics in test as shown in (Table 3-5).

In addition to the reasons above, many factors lie beneath the ability of bacteria to resist antibiotics, one of which is the biofilm. In this study, the biofilm forming bacteria displayed significantly high antibiotic resistance, which was correspondent to other studies (Smith and Hunter, 2008, Corona and Martinez, 2013). In the biofilm state, the antibiotics were more difficult to diffuse into the bacteria and the compounds of the matrix bound to the antibiotics also increased the difficulty (Corbin *et al*., 2011). The extracellular DNA display antibiotic chelating activity (Lewis, 2010). In addition to different metabolic state as a result of their grade of nutrients and oxygen viability depend on their depth inside the biofilm layers which affect bacterial susceptibility to antibiotics (Corona and Martinez, 2013). Gene transfer also plays a major role in antibiotic resistant by sharing genetic information via horizontal gene transfer among bacterial cells biofilm involvement antibiotic resistant capability (Bagge *et al*., 2004).

The most effective antibiotics on biofilm bacteria were azithromycin on *S. lentus* and imipenem on gram negative bacteria (*S. fonticola* and *Pantoea* sp.) so it chosen to complete the MIC testing manually.

3.4.2. MIC testing:

MIC values were determined by macrodilution broth method to selected antibiotics (azithromycin and imipenem) for tested biofilm bacteria (*S. lentus*) (Table 3-6) and (*S. fonticola* and *Pantoea* sp.) (Table 3-7) respectively. All MIC values referred to sensitivity of these isolates to mention antibiotics.

Table (3-6): The MIC value of azithromycin to *S. lentus* isolates or minimum biofilm inhibitory concentration.

Bacteria	MIC (µg/ ml)	Interpreted
S. lentus 1	2	S
S. lentus 2	2	S
S. lentus 3	2	S

S=sensitive

Table (3-7): The MIC value of imipenem to *S. fonticola* and *Pantoea* sp. isolates.

Bacteria	MIC (µg/ ml)	Interpreted
S. fonticola 1	2	I
S. fonticola 2	2	I
S. fonticola 3	2	I
Pantoea sp. 1	1	S
Pantoea sp.2	1	S
Pantoea sp.3	1	S
Pantoea sp.4	0.5	S

I= intermediate; S= sensitive

The MIC product solution was later use in real time (RT) gene expression analysis to evaluate the antibiotics effect on expression of biofilm genes to selected bacteria.

Because of biofilm bacteria have multi-drugs resistant ability, it is urgent need to develop a novel method to kill biofilm bacteria. Recently, many research focus on antibacterial activity of silver nanoparticles (Chojniak *et al*., 2017, Erjaee *et al*., 2017).

3.5. Characterization of silver nanoparticles:

3.5.1. Visual inspection:

Chemical SNPs: In chemical synthesis, $AgNO_3$ solution and sodium borohydride were colorless before mixing together, silver nitrate was converted to brown color when added as drops to sodium borohydride as shown in (Fig. 3-14) and the color was increase in degree

to be dark brown when continue mixing with magnetic stirrer for a half one hour.

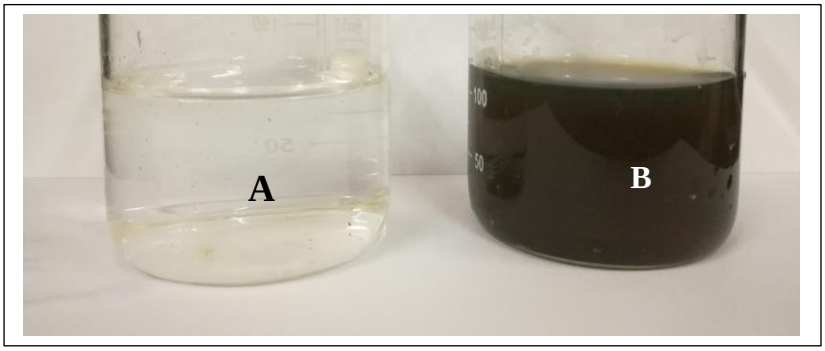


Figure (3-14):Visual observation of change in color in chemical synthesis, A= $AgNO_3$ solution; B= SNPs solution.

Biological silver nanoparticles: *Enterobacter* cell free filtrate solution was pale yellow in color then converted to brown color when added silver nitrate solution and after 24hrs incubation in shaker incubator at 150 rpm as shown in (Fig. 3-15) and that reduction indicator to convert Ag^{+} to Ag^{0} to be nanoparticles. Chang in color were previously reported in many extracellular synthesis method from bacterial source (Wang *et al*., 2016) and from *Enterobacter cloacae* specially (Shahverdi *et al*., 2007).

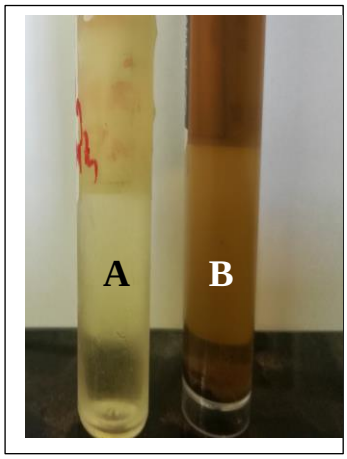


Figure (3-15): Visual observation of change in color in biosynthesis, A= cell free supernatant solution; B= SNPs solution

The brown color in both methods related to excitation of SNPs surface Plasmon vibration (Kumar *et al*., 2015) as a result of SNPs production.

3.5.2. **UV-Vis spectroscopy**:

In Chemical SNPs UV absorption spectrophotometer revealed the absorption band was at 390 nm (Fig. 3-16) due to SNPs Plasmon resonance. The absorption energy of SNPs depend on Plasmon resonance degree which represent the ratio of silver ion to silver zero valent (Mehr et al., 2015).

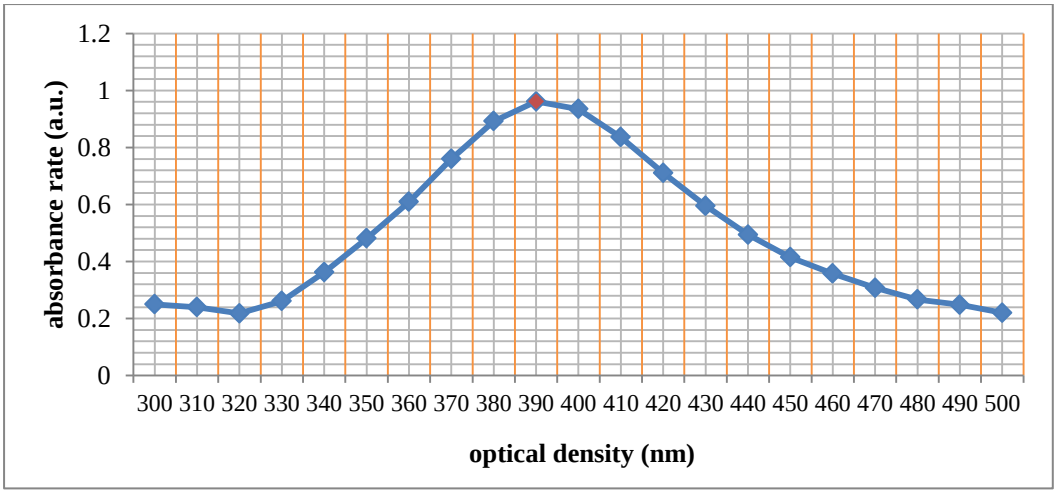


Figure (3-16): UV-Vis spectrophotometer analysis of chemical silver nanoparticles synthesis.

While the observation of biological SNPs synthesis by *E. cloacae* was strong with narrow surface peak reach to 400 nm (Fig. 3-17). Narrow peak indicate to narrow size range of nanoparticles less than100 nm (Shahverdi *et al*., 2007).

UV-vis spectra absorbance of SNPs was due to surface plasmon resonance absorption because of vibration combination of SNPs free electrons with light wave and also it was consider as other indicator of

nanoparticles production at the range of absorption from 390-420 nm (Gurunathan *et al*., 2014).

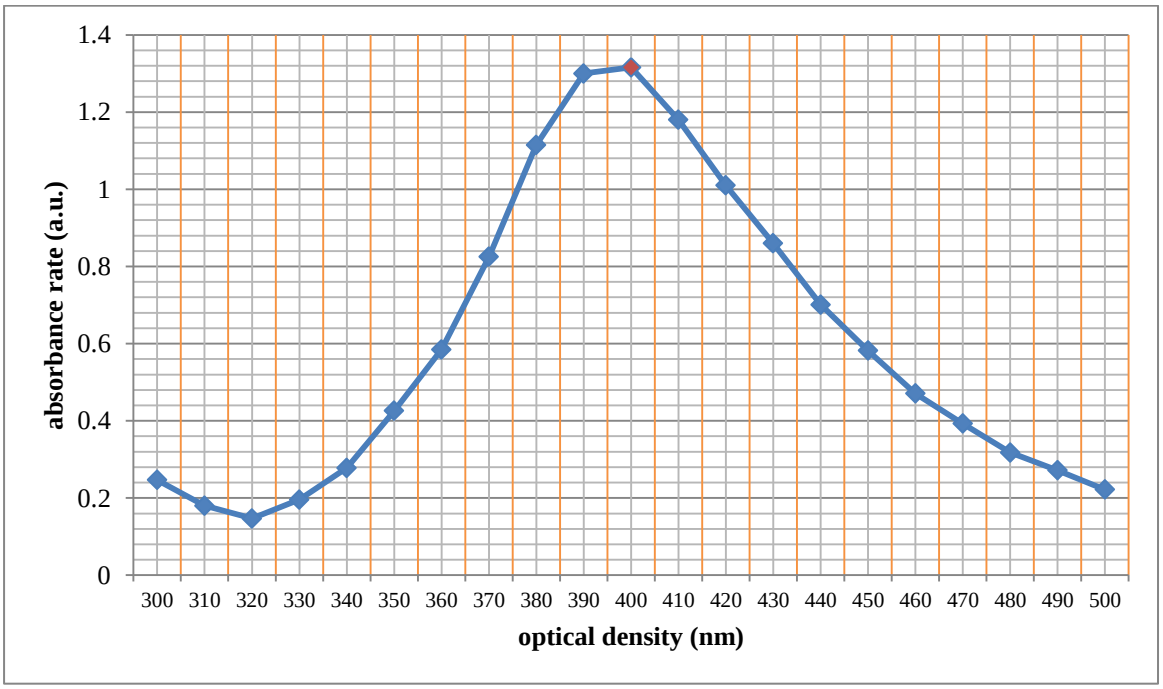


Figure (3-17): UV-Vis spectrophotometer analysis of biological silver nanoparticles synthesis.

3.5.3. Fourier Transform Infrared Spectroscopy (FTIR):

The results showed in (Fig. 3-18 and 3-19) illustrated Fourier Transform-Infrared (FTIR) spectrum of chemical and biological synthesized SNPs respectively in the wavelength range from 500 cm^{-1} to 4000 cm^{-1}. It can be shown in the figure that the FTIR spectrum presented absorption bands at 1635, 1636 and 3330, 3445 cm^{-1} in chemical and biological synthesis respectively. The band at 1636 and1635 cm^{-1} refers to vibration stretching group C=O indicating the carboxylic acids group which bounded to silver nanoparticles. The peak at 3445 and 3330 cm^{-1} was belonged to O-H stretching vibration band for polyphenolic compounds in the extract adsorbed on the surface of biological

synthesized nanoparticles as well as the presence of water into the system and thereby intermolecular Hydrogen-bonding in case of chemically synthesized silver nanoparticles. All these stretching vibrations bands clarify that carboxylic groups and polyphenolic groups are coated on the surface of silver nanoparticles producing stabilized nanoparticles with negligible aggregation.

FTIR spectra were analyzed to detect biomolecules that involve in Ag+ reduction (Pasupuleti *et al*., 2013).

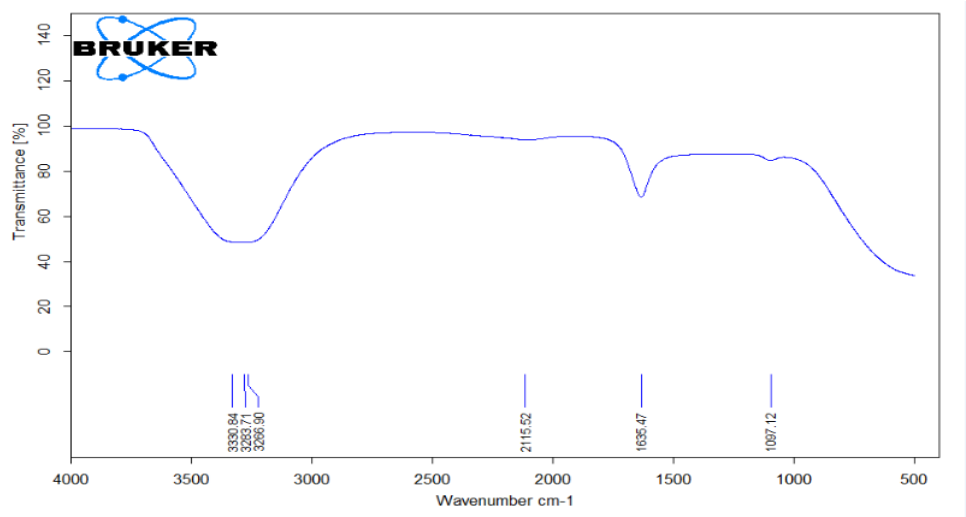


Figure (3-18): FTIR spectrum of SNPs, chemical synthesis with distinct peaks.

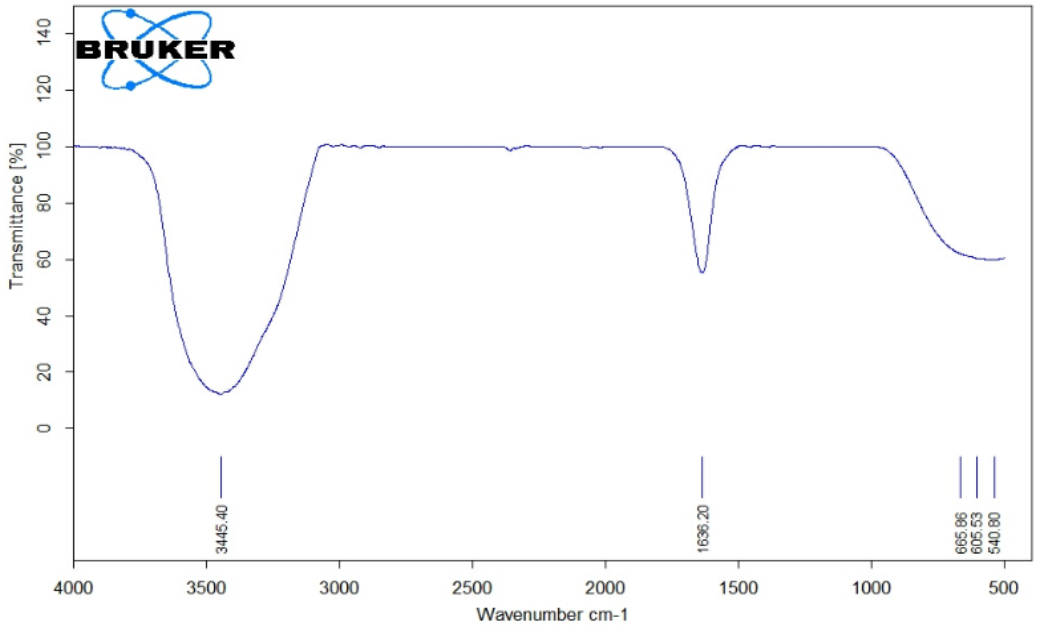
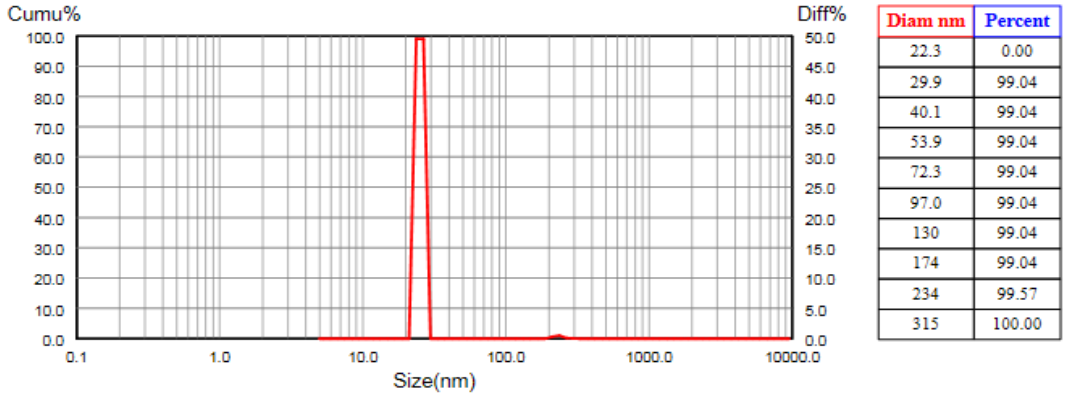


Figure (3-19):FTIR spectrum of SNPs, biological synthesis with distinct peaks.

3.5.4. Size analyzer:

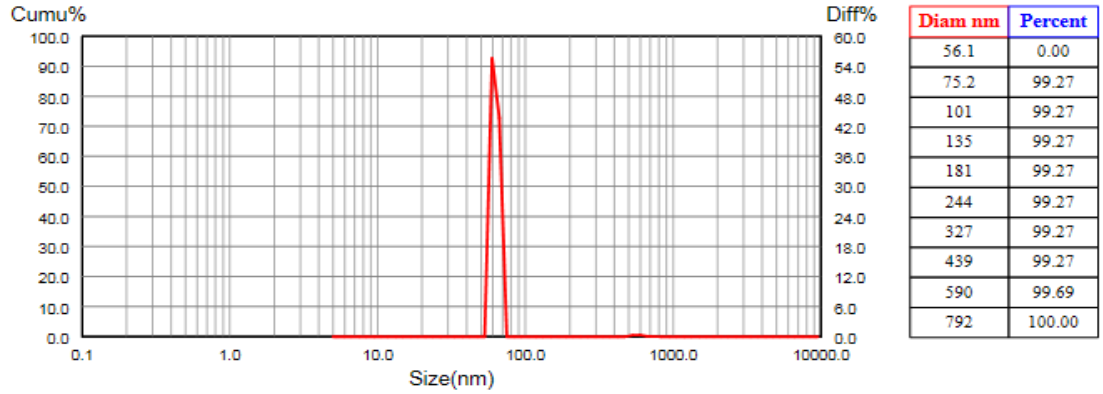
SNPs size of both methods were determined by dynamic light scattering. The size of nanoparticles distribution analysis of chemical method revealed the average of particles size was approximately 22-28nm (25± 3) (Fig. 3-20) while the biosynthesis SNPs was 56-70nm (63± 7) (Fig. 3-21).

The antibacterial activity of SNPs influence of its size particles, the small particles well known more effective than large one as antibacterial and antibiofilm (Ghotaslou *et al*., 2017). Many previous studies reported that antibacterial activity based on particles size of SNPs (Martinez-Castanon *et al*., 2008).



Diam nm	Percent
22.3	0.00
29.9	99.04
40.1	99.04
53.9	99.04
72.3	99.04
97.0	99.04
130	99.04
174	99.04
234	99.57
315	100.00

Figure (3-20): Size distribution analysis of chemical SNPs particle size was approximately 25 nm.



Diam nm	Percent
56.1	0.00
75.2	99.27
101	99.27
135	99.27
181	99.27
244	99.27
327	99.27
439	99.27
590	99.69
792	100.00

Figure (3-21): Size distribution analysis of biological SNPs particle size was approximately 63 nm.

3.5.5. Scanning electron microscope examination (SEM):

The (Fig. 3-22, 3-23) revealed typical SEM micrograph of chemical and biological SNPs obtained by the reduction of $AgNO_3$ solution with sodium brohydred and cell-free filtrate of *E. cloacae* respectively. The morphology of NPs was cubic in shape, uniformly (mono dispersed) without significant aggregation in both types. The particle size was ranged from 22-28 nm to chemical SNPs and 56-72 nm to biological SNPs.

Scanning electron microscopy has been employed to determine the shape, size and morphology of chemical and biological synthesized SNPs.

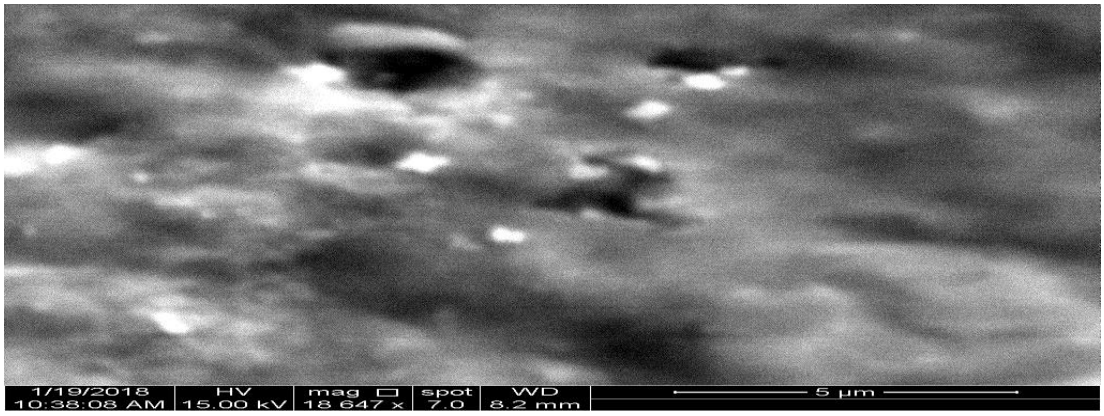


Figure (3-22): SEM micrograph of chemosynthesis SNPs. The image shows size and cubic shape of SNPs

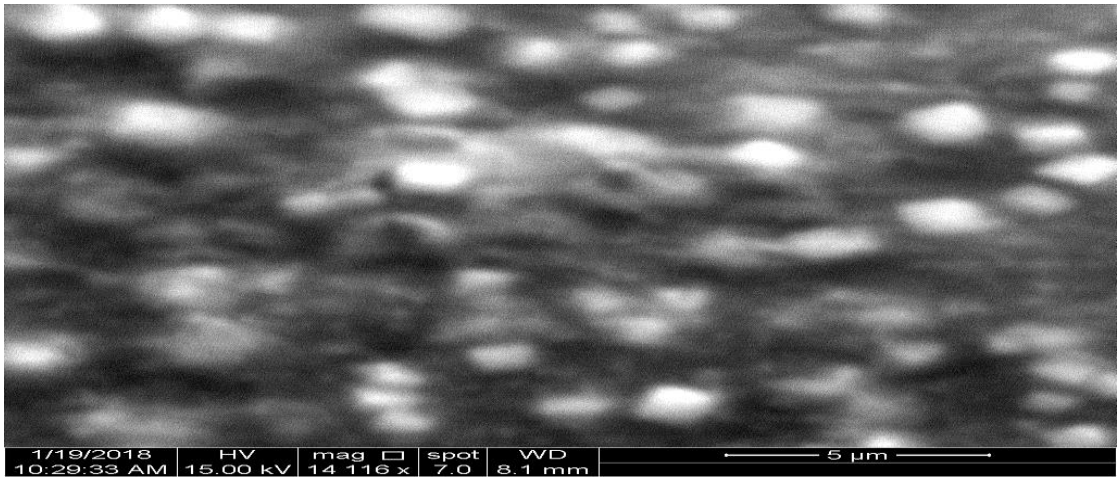


Figure (3-23): SEM micrograph of biosynthesized SNPs. The image shows size and cubic shape of SNPs

3.6. Antimicrobial effects of SNPs on biofilm bacteria:

Antimicrobial activity of the chemo and biosynthesized SNPs was evaluated using the agar well diffusion and MIC method on selected biofilm forming bacteria (*S. lentus*, *S. fonticola* and *Pantoea* sp.).

3.6.1. Agar well diffusion method:

1. ***S. lentus:***

The antimicrobial effect of both types SNPs on *S. lentus* and CoNS as control were shown in (Fig. 3-24) to chemical SNPs and (Fig. 3-25) to biological SNPs.

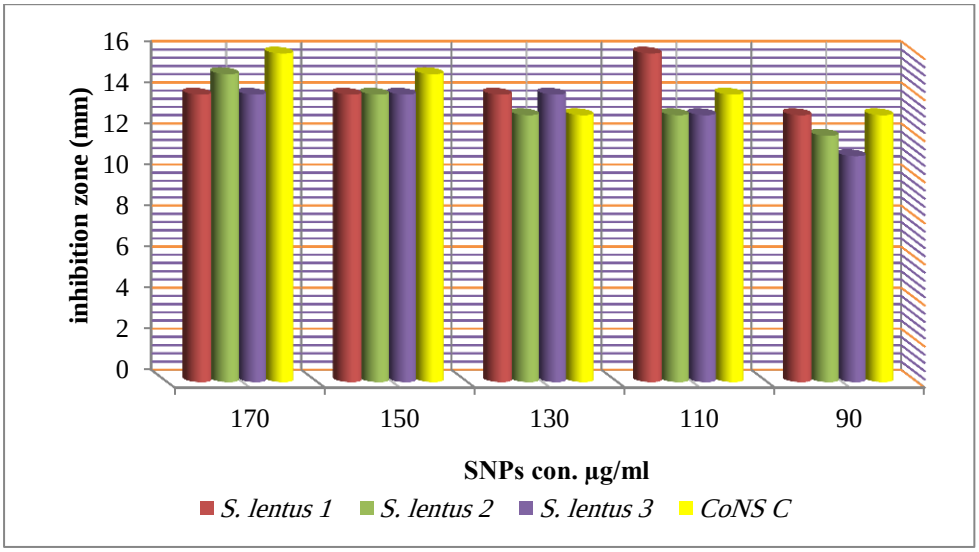


Figure (3-24): Zone of inhibition (mm) of chemical SNPs against *S. lentus* and CoNS as control.

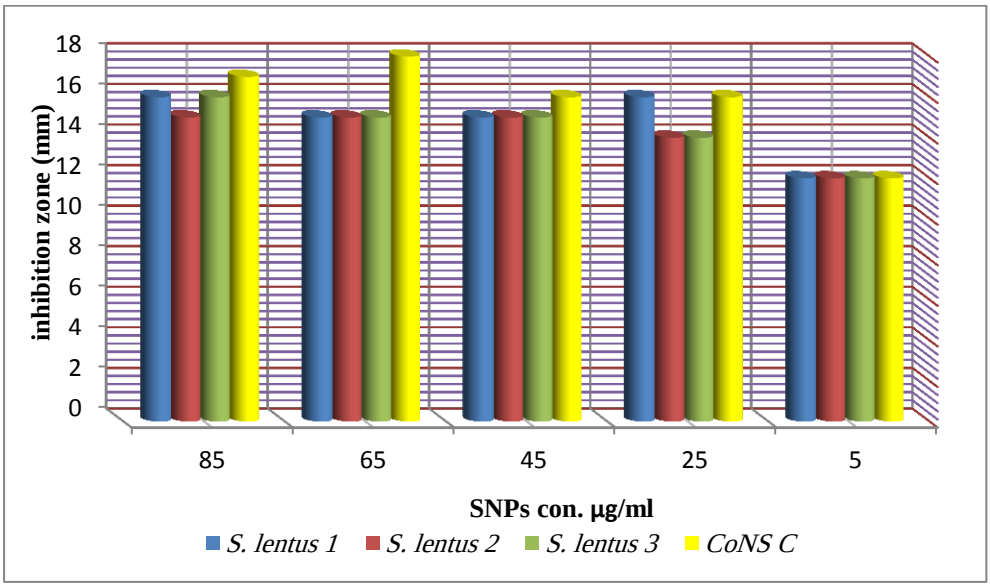


Figure (3-25): Zone of inhibition (mm) of biological SNPs against *S. lentus* and CoNS as control.

2. *S. fonticola:*

The antimicrobial effect of both types SNPs on *S. fonticola* and *Serratia* sp. as control were shown in (Fig 3-26) to chemical SNPs and (Fig 3-27) to biological SNPs.

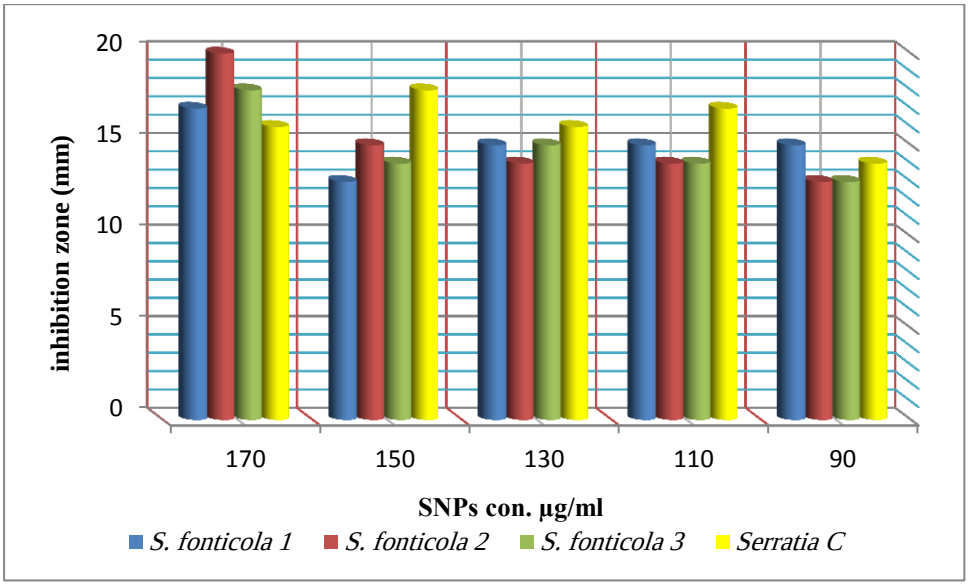


Figure (3-26): Zone of inhibition (mm) of chemical SNPs against *S. fonticola* and *Serratia* sp. as control.

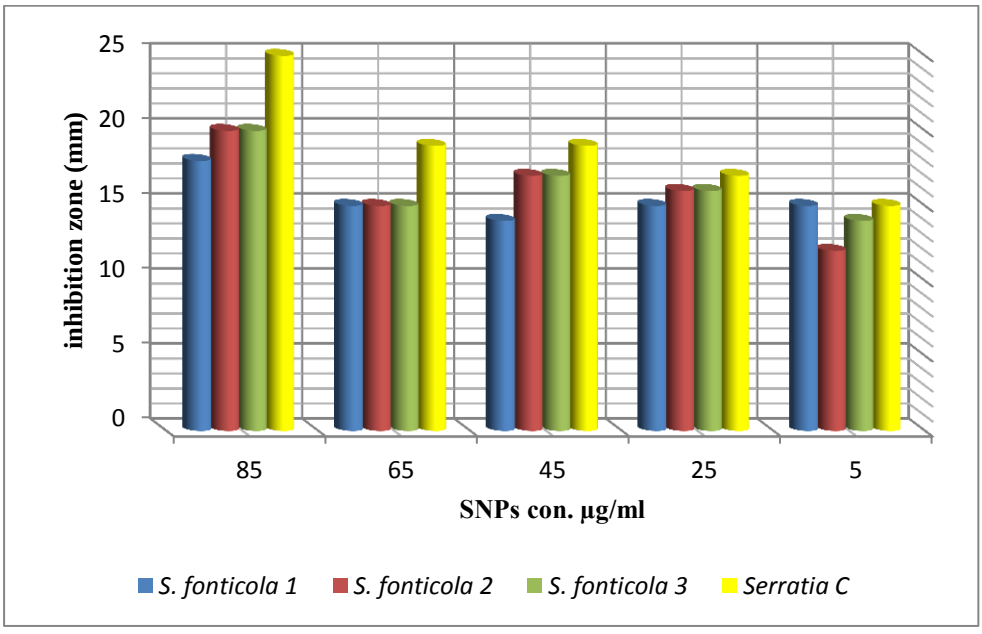


Figure (3-27): Zone of inhibition (mm) of biological SNPs against *S. fonticola* and *Serratia* sp. as control.

3. *Pantoea* sp.:

The antimicrobial effect of both types SNPs on *Pantoea* sp. and *E. coli* as control were shown in (Fig 3-28) to chemical SNPs and (Fig 3-29) to biological SNPs.

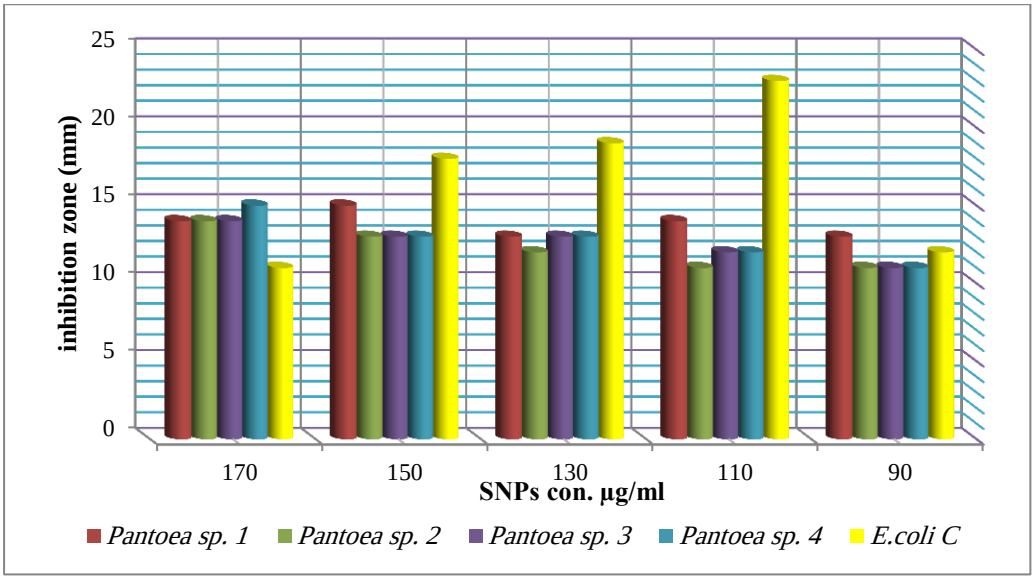


Figure (3-28): Zone of inhibition (mm) of chemical SNPs against *Pantoea* sp. and *E. coli* as control.

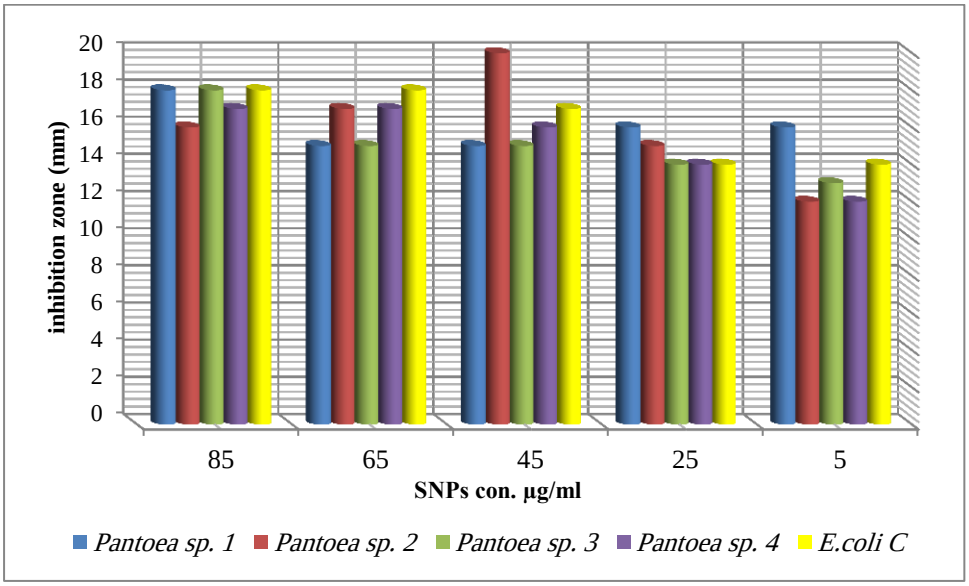


Figure (3-29): Zone of inhibition (mm) of biological SNPs against *Pantoea* sp. and *E. coli* as control.

Antibacterial activity of SNPs were evaluated by many previous studies (Chaloupka *et al*., 2010, Gade *et al*., 2010) but less studies focus

on anti-biofilm activity of SNPs such as (Mathur et al., 2006, Guzmán et al., 2009) and comparative effect of both types (chemical and biological SNPs).

The antimicrobial effect of the silver and silver nanoparticles may related to attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell (Kvitek *et al*., 2008). It is also possible that silver and silver nanoparticles not only interact with the surface of membrane, but can also penetrate inside the bacteria (Morones *et al*., 2005). Many researchers also proposed that Ag+ ions interact with the thiol groups in bacteria proteins, affecting the replication of DNA(Marini *et al*., 2007). It has been reported that Ag+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane (Holt and Bard, 2005).

SNPs were recorded to be anti- biofilm agents which effective against biofilm bacteria formation, also most effective against multi- drug resistance bacteria resulted from biofilm formation to solve most serious problem to worldwide public health (Ansari *et al*., 2011, Ghotaslou *et al*., 2017, Shaker and Shaaban, 2017).

From statistical analysis of antimicrobial results (Table 3-7) conclude that biosynthesis SNPs more effective on biofilm bacterial isolates gram positive and negative than chemosynthesis SNPs, with significant differences $p<0.001$ among *Pantoea* sp. isolates and no significant differences $p= (0.229, 0.079)$ among *S. fonticola* and *S. lentus* isolates at respectively, although less concentration and larger particle size (63nm) of biosynthesis SNPs used in experiment than chemosynthesis SNPs concentration and particle size (25nm). Some

studies approved that biosynthesis SNPs had a potent anti-biofilm activity than chemosynthesis (Chojniak *et al*., 2017).

The results revealed also that biofilm gram positive bacteria (*S. lentus*) was less susceptible to chemo and biosynthesis SNPs than biofilm gram negative (*S. fonticola* and *Pantoea* sp.). Many studies supports that results were gram positive bacteria less susceptible to SNPs than gram negative bacteria such as (Gurunathan *et al*., 2014, Erjaee *et al*., 2017, Shaker and Shaaban, 2017) and that may related to cell wall differences structure between gram positive and negative. Gram positive bacteria cell wall more thicker than gram negative bacteria because it has thicker layer of peptidoglycan compared to gram negative cell wall and present of teichoic acids molecules make it more strong to sequester silver ions, so the gram positive bacteria less introduce silver ions to making fatal change (Pal *et al*., 2007, Egger *et al.*, 2009) therefore gram positive less affect than gram negative bacteria. Control strain non- biofilm (CoNS) were more susceptible to SNPs than biofilm staphylococci (*S. lentus*) agree with other study which found non- biofilm staphylococci more susceptible than biofilm strain (Ansari *et al*., 2011). In chemical and biological SNPs there was no significant differences among *S. lentus* and control isolates at p-value= (0.395, 0.884) respectively (Table 3-8).

Gram negative biofilm bacteria (*S. fonticola* and *Pantoea* sp.) was more susceptible to SNPs in both types. It was thought that SNPs cause pits in gram negative cell wall leading to increase permeability of cell membrane and inhibit respiratory chain lead to kill treated bacterial (Siegel *et al*., 2007, Paredes *et al*., 2014).

The results revealed that the antibacterial activity of SNPs increased directly with concentration increasing to both types. The

inhibition zone diameter of tested biofilm bacteria increased significantly with increasing the concentration of SNPs. Tiwari *et al*. pointed that protein leakage from bacterial cell treated with SNPs increased along with SNPs concentration which lead to cell death, that releasing of protein was lower in gram positive than negative bacteria (Tiwari et al., 2008). Statistical analysis to antimicrobial effect of chemical SNPs recover that was no significant differences p=0.229 among *S. fonticola* isolates and control and p= 0.171 among *Pantoea* sp. isolates and its control and no significant differences p= (0.254, 0.959) in biological SNPs respectively (Table 3-9).

Table (3-8): Statistical analysis (t test) of antimicrobial effect chemical and biological SNPs:

Bacteria	Chemical SNPs IZ± SE	Biological SNPs IZ± SE	p-value
S. fonticola	15.000±0.507	15.933±0.564	P=0.229
Pantoea sp.	12.850±0.283	15.550±0.450	*****P<0.001
S. lentus	13.600±0.305	14.466±0.363	P=0.079

*Significant differences, IZ= inhibition zone, SE= stander error

Table (3-9): Statistical analysis (t test) of antimicrobial effect of SNPs in both types on biofilm bacterial isolates and control

Biological SNPs	Bacteria IZ± SE	Control IZ± SE	p-value
S. fonticola	15.933±0.564	17.400±1.029	P=0.254
Pantoea sp.	15.550±0.450	15.600±0.678	P=959
S. lentus	14.466±0.363	14.600±2.607	P=0.884
Chemical SNPs			
S. fonticola	15.00±0.507	16.200±0.663	P=0.229
Pantoea sp.	12.850±0.283	16.600±2.249	P=0.171
S. lentus	13.600±0.305	14.200±0.583	P=0.395

IZ= inhibition zone, SE= stander error

3.6.2. MIC method:

The macrodilution method to determine MIC value of both SNPs types to each tested bacteria, the results show the MIC values of biological SNPs to (*S. lentus, S. fonticola* and *Pantoea* sp. isolates) were 25 µg/ml while chemical SNPs MIC values were 90 µg/ml to all isolates. These values solution were used later in RT PCR analysis to evaluate the gene expression among tested bacteria.

3.7. Effect of Combination between antibiotics and SNPs on biofilm bacteria: it was done by the following:

1. Agar well diffusion method:

The results showed that antibacterial activity of SNPs in two types in combination with both antibiotics were increased and enhanced on tested biofilm bacteria inhibited growth according to (Fig. 3- 30, 3-31, 3-32, 3-33, 3-34, 3-35). The antibacterial effect of combination SNPs and antibiotic exhibited higher than SNPs or antibiotic alone, this result corresponded with (Gurunathan, 2014, Gurunathan *et al*., 2014). Moreover those researchers pointed out that combined antibiotics with SNPs make it as greater anti- biofilm activity and elevated bacterial cell death level, so treatment with combination of antibiotics and SNPs consider more potent effectiveness as antibacterial and ant- biofilm.

Namasivayam *et. al*, concluded that SNPs made a good compatibility in combination with antibiotics to inhibit bacterial biofilm (Namasivayam *et al*., 2012). Therefore SNPs can be used as adjuvants to antibiotics when combined with it by enhancing antibiotics activity against gram positive and negative bacteria (Gurunathan, 2014). Recently reported that combination between SNPs and Vancomycin increase inhibit biofilm activity to 55% and 75% to gram positive and negative bacteria respectively (Gurunathan *et al*., 2014). Statistical analysis recover that biological SNPs in combination with imipenem more effective than

chemical SNPs with imipenem with significant differences at p =0.005 and 0.028 for *S. fonticola* and *Pantoea* sp. respectively but there were no significant differences p = 0.562 in *S. lentus* bacteria between chemical and biological SNPs with azithromycin (Table 3-10)

Table (3-10): Statistical analysis (t test) of antimicrobial effect of combination between SNPs in both types with imipenem and azithromycin.

Bacteria	Chemical SNPs+ imipenem (IZ± SE)	Biological SNPs + imipenem(IZ± SE)	p-value
S. fonticola	19.571±1.237	25.800±1.718	*P=0.005
Pantoea sp.	17.071±0.778	19.950±1.03	*P=0.028
	Chemical SNPs+ azithromycin (IZ± SE)	Biological SNPs + azithromycin(IZ± SE)	
S. lentus	18.142±0.865	19.000±1.238	P=0.562

*Significant differences, IZ= inhibition zone, SE= stander error

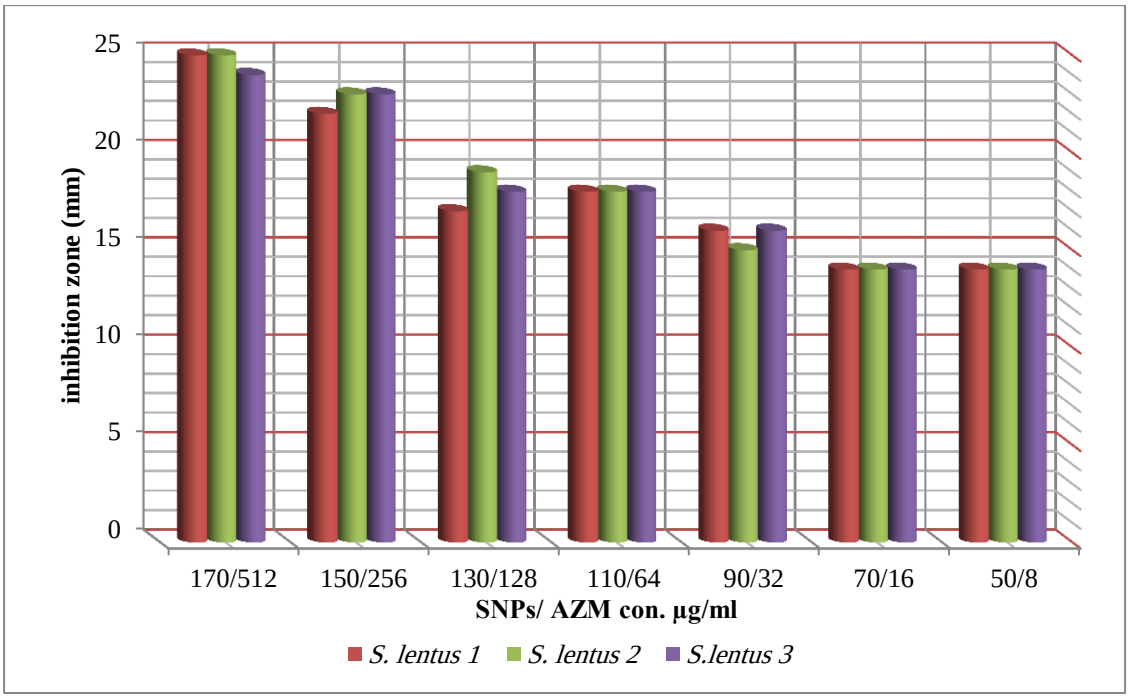


Figure (3-30): Combination antibacterial effect of AZM and Chemical SNPs on biofilm forming *S. lentus*.

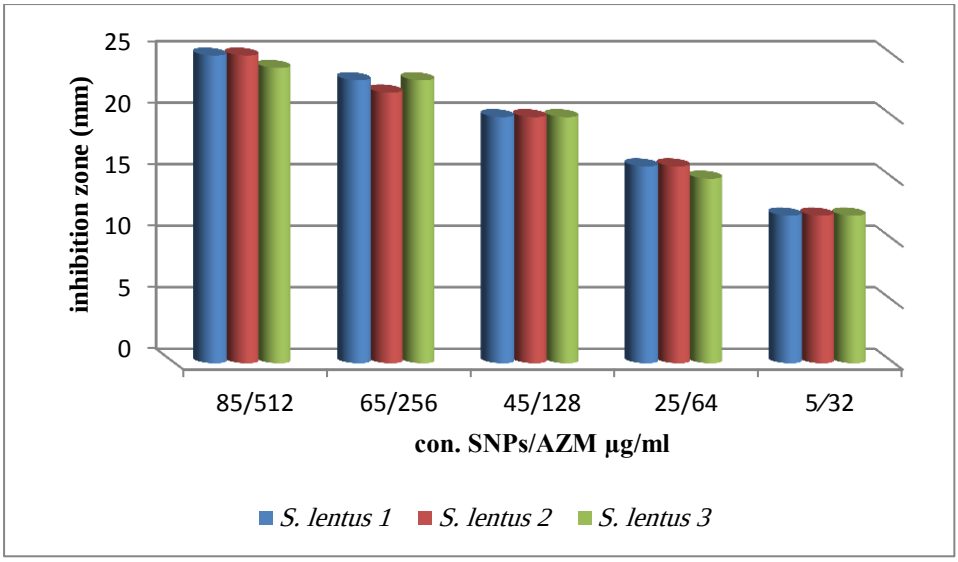


Figure (3-31): Combination antibacterial effect of AZM and biological SNPs on biofilm forming *S. lentus*.

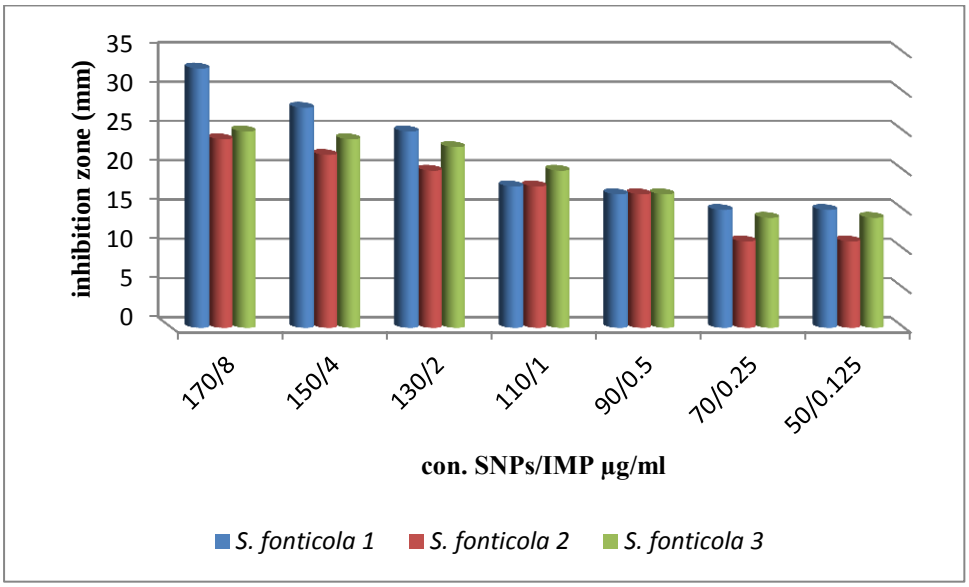


Figure (3-32): Combination antibacterial effect of imipenem and chemical SNPs on biofilm forming *S. fonticola* .

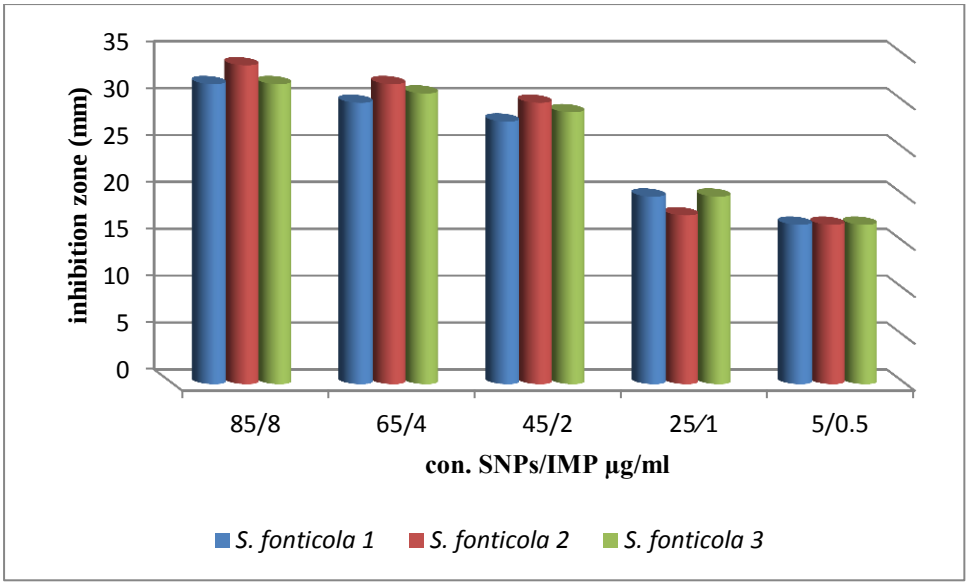


Figure (3-33): Combination antibacterial effect of imipenem and biological SNPs on biofilm forming *S. fonticola*.

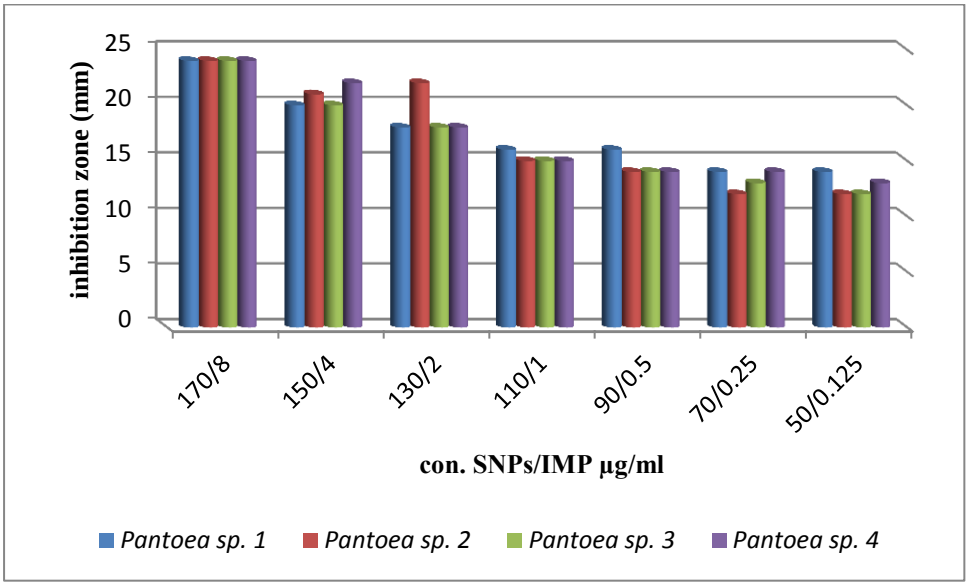


Figure (3-34): Combination antibacterial effect of imipenem and chemical SNPs on biofilm forming *Pantoea* sp.

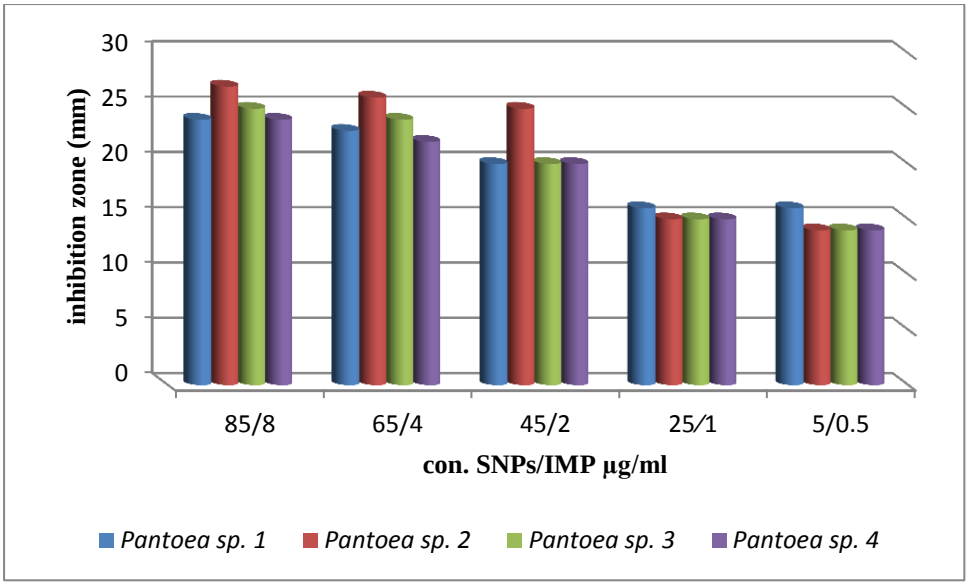


Figure (3-35): Combination antibacterial effect of imipenem and biological SNPs on biofilm forming *Pantoea* sp.

The results of combination SNPs in two types with both antibiotics, revealed the effect to be a synergism effect. Where the (Table 3-11, 3-12, 3-13 and 3-14) shows a highly synergism effect and anti- biofilm activity mostly more than each one alone and to both types (chemical and biological) and to all tested biofilm bacteria. Anti-biofilm activity of SNPs in other studies increased to reach 50% and 70% to gram positive and negative biofilm forming bacteria respectively when combination with ampicillin (Gurunathan *et al*., 2014). In addition, it increases bacterial susceptibility to antibiotics when combined with them as synergistic effect especially in biofilm infection like nitrofurazone increased its effect in silver present (Kostenko *et al*., 2010)

Table (3-11): Synergism effect % between chemical SNPs and azithromycin on biofilm *S. lentus* isolates:

Con.	*S. lentus 1*	*S. lentus 2*	*S. lentus 3*
170/512	78.5	66.6	71.4
150/256	57	64.2	64.2
130/128	21.4	46.1	28.5
110/64	12.5	38.4	38.4
90/32	23	25	45.4

Table (3-12): Synergism effect % between chemical SNPs and imipenem on biofilm *S. fonticola* and *Pantoea* sp. isolates:

Con.	*S. fontic 1*	*S. fonti2*	*S. fonti 3*	*Pantoea 1*	*Pantoea 2*	*Pantoea 3*	*Pantoea 4*
170/8	94	20	38.8	71.4	71.4	71.4	60
150/4	115	46	71.4	33.3	61.5	53.8	69.2
130/2	66.6	42	53.3	28.5	83.3	38.4	38.4
110/1	20	38	28.5	14.2	36.3	7.1	25
90/0.5	13	30.7	30.7	23	27.2	27.2	27.2

Table (3-13): Synergism effect % between biological SNPs and azithromycin on biofilm *S. lentus* isolates:

Con.	*S. lentus 1*	*S. lentus 2*	*S. lentus 3*
85/512	56.2	66.6	50
65/256	53.3	46.6	53.3
45/128	33.3	33.3	33.3
25/64	0	14.2	6
5/32	0	0	0

Table (3-14): Synergism effect % between biological SNPs and imipenem on biofilm *S. fonticola* and *Pantoea* sp. isolates:

Con.	*S. fonti1*	*S. fonti2*	*S. fonti 3*	*Pantoea 1*	*Pantoea 2*	*Pantoea 3*	*Pantoea4*
85/8	77.7	70	60	33.3	68.7	38.8	41.1
65/4	100	113	106	53.3	52.9	60	29.4
45/2	100	76.4	93.3	33.3	25	33.3	25
25/1	33.3	12.5	17.6	0	0	7.1	7.1
5/0.5	13.3	41.6	21.4	0	16.6	7.1	16

3.8. Real time gene expression:

Biofilm formation is cooperative genetic process required different genes to cause and regulate biofilm formation. Nanoparticles may

potentially effect on these process so the goal is to evaluate the silver nanoparticles effect on gene expression level of biofilm causative and regulatory genes. Depending on the previous literature about the gene expression of biofilm for the bacterial isolates under study, there was no studies are found to deal this subject except chines study when used chitosan to inhibit staphylococci biofilm formation by down-regulate *icaA* gene expression (Tan *et al*., 2012), so this study is considered the first one that applicated the SNPs prepared chemically and biologically to minimize the biofilm formation in bacteria caused the catheterization patient. For this reason, the discussion were limited to interpretation the recent results.

3.8.1. RNA extraction and purity:

The results of total RNA was estimated by Nanodrop spectrophotometer as shown in (Table 3-15) (*S. lentus, S. fonticola* and *Pantoea* sp.), which explain that the total RNA concentration ranged (384.6-634.3ng/µl) and the RNA purity at ratio of 260/280 nm ranged (1.71-1.89). These data emphasize that the technique used for RNA extraction was perfect for amplification in RT-PCR System. Total RNA was extracted by using (Total RNA extraction Trizol kit) in performing (RT-qPCR) for relative gene expression analysis of biofilm genes (*icaA, smaI* and *esaL*) for tested and control of (*S. lentus, S. fonticola and Pantoea* sp.) respectively in different treatments.

The concentration and purity of extraction RNA have powerful influence on gene expression outcome and determine the accuracy of RNA profile. RNAs characterized by sensitivity molecules in compared to DNAs, which was easily denaturized by heating , UV or nuclease, so RNA sample should be free of protein, nucleases and inhibitor enzyme or

contaminated with DNA molecules (Hellemans and Vandesompele, 2014).

Table (3-15): Total RNA extraction of test and control of biofilm bacterial isolates with different treatments.

Treatment type	Con. (ng/uL)	Purity 260/280nm
Treat with chemical SNPs		
S. fonticola 1	54.21	2.35
S. fonticola 2	55.24	2.66
S. fonticola 3	52.43	2.41
Pantoea sp. 1	52.33	2.67
Pantoea sp. 2	55.26	2.55
Pantoea sp. 3	54.64	2.25
Pantoea sp. 4	52.34	2.44
S. lentus 1	53.86	2.66
S. lentus 2	55.22	2.49
S. lentus 3	54.31	2.57
Treat with Biological SNPs		
S. fonticola 1	56.11	2.55
S. fonticola 2	52.34	2.64
S. fonticola 3	52.53	2.36
Pantoea sp. 1	82.23	2.22
Pantoea sp. 2	52.76	2.41
Pantoea sp. 3	54.60	2.53
Pantoea sp. 4	52.44	2.67
S. lentus 1	54.79	2.58
S. lentus 2	55.32	2.68
S. lentus 3	56.21	2.56
Treat with Imipenem		
S. fonticola 1	34.23	2.18
S. fonticola 2	63.45	2.22
S. fonticola 3	55.64	2.30
Pantoea sp. 1	32.78	2.19
Pantoea sp. 2	68.18	2.33
Pantoea sp. 3	54.33	2.52
Pantoea sp. 4	62.77	2.31
Treat with Azithromycin		
S. lentus 1	78.84	2.41
S. lentus 2	76.54	2.63
S. lentus 3	78.80	2.10
Control		
S. fonticola 1	56.34	2.64
S. fonticola 2	84.78	2.31

S. fonticola 3	65.22	2.51
Pantoea sp. 1	43.46	2.64
Pantoea sp. 2	78.41	2.33
Pantoea sp. 3	55.23	2.43
Pantoea sp. 4	64.12	2.15
S. lentus 1	78.45	2.64
S. lentus 2	72.44	2.43
S. lentus 3	75.21	2.50

The rest combination treatments data of RNA concentration and purity were documented in (Appendix 25).

3.8.2. Quantitative real-time PCR analysis:

The combination of reverse transcription with PCR to be (RT-PCR) made a powerful gene expression quantification method (Livak and Schmittgen, 2001). To gain an insight into molecular estimation differences in gene expression of biofilm regulation *icaA, smaI* and *esaL* genes in test and control of *S. lentus, S. fonticola and Pantoea* sp. isolates respectively, quantitative Reverse Transcription Real-Time PCR (RT-qPCR) was performed .

The gene expression patterns were evaluated in biofilm bacterial genes before and after treatment with antibiotics and SNPs alone and combination between them by normalization with expressed of housekeeping gene (reference gene), RNA polymerase β subunit gene (*rpoB*). Reference gene was potent to assess the accuracy of tested gene (Li *et al*., 2018) and must to be expressed constantly according to experimental conditions (Livak and Schmittgen, 2001). All that analysis by RT-PCR was considered a standard analysis technique to assess gene expression of potential gene which impact on pathological diseases (Fu *et al*., 2006).

The current study investigated the expression stability of (*rpob*) housekeeping genes which highly conserved RNA subunit as reference genes to study gene expression in bacterial biofilms (*S. lentus*, *S. fonticola* and *Pantoea* sp.) to biofilm regulated and causative genes (*icaA*, *smaI* and *esaL*) and determine the differences in gene expression of biofilm gene after and before treated with SNPs and antibiotics.

3.8.2.1. Relative quantification:

Relative quantification mean the change of target gene expression relative to control group (Livak and Schmittgen, 2001). Quantification PCR estimate the amplification of sampling DNA at cycle number according to induce of fluorescence indicator (SYPRgreen), which was used as amplification detection of target genes and expression levels assessment (Fu *et al*., 2006). The figures (3-36, 3-37, 3-38, 3-39, 3-40) show the RT- qPCR amplification biofilm genes to tested bacteria and control.

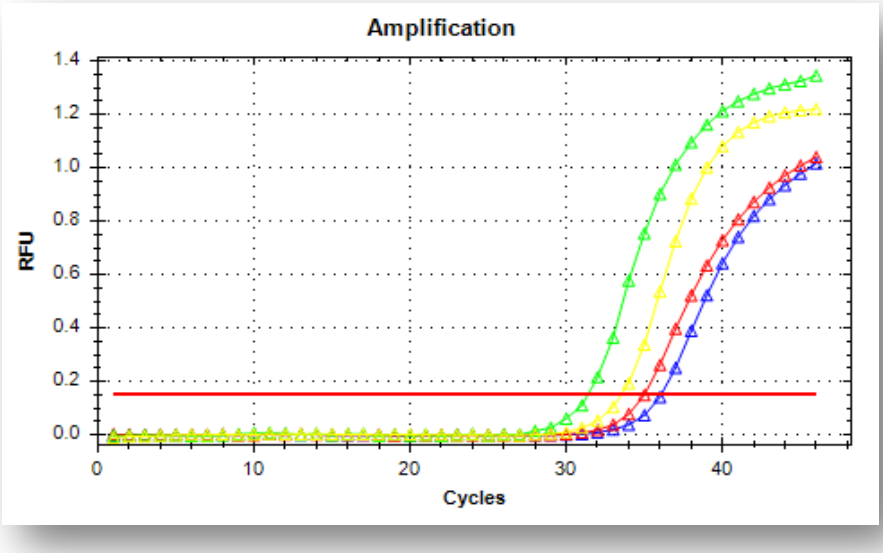


Figure 3-36 : RT- qPCR amplification biofilm formation genes (*icaA*) in treated and untreated (control) *S. lentus* where red blot= T1(chemical SNPs), blue plot=

T2(biological SNPs), yellow plot=T3 (azithromycin) and green plot= control, red line = threshold line.

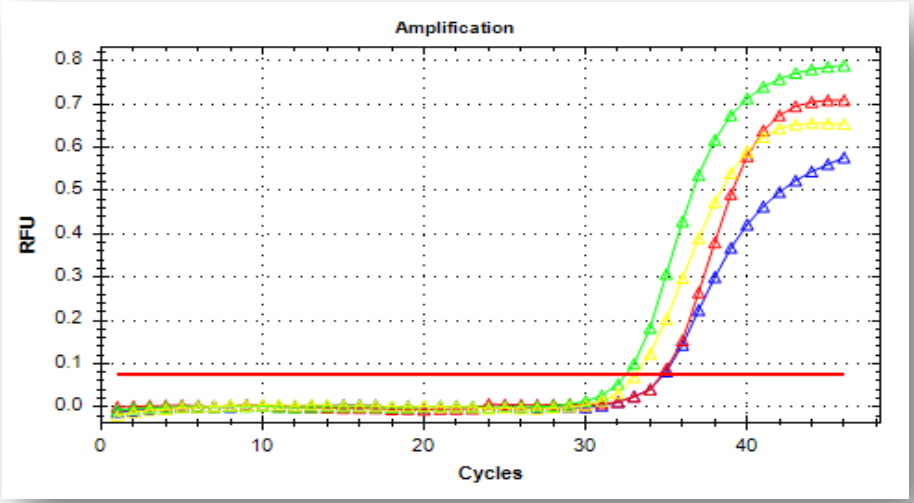


Figure 3-37: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola* 1,2 where red blot= T1(chemical SNPs), blue plot= T2(biological SNPs), yellow plot=T3(imipenem) and green plot= control, red line = threshold line.

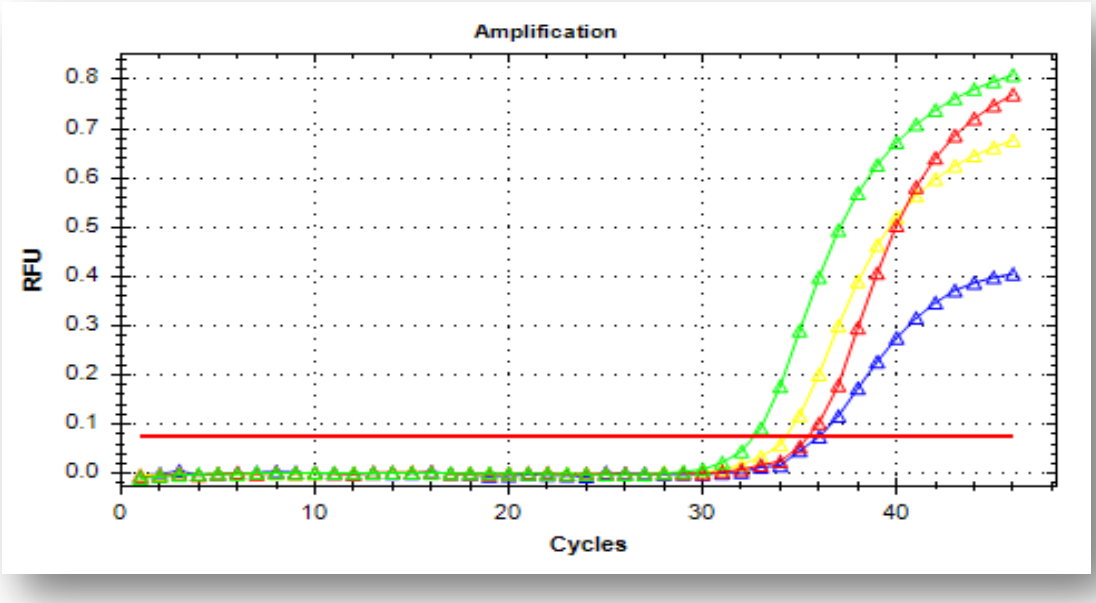


Figure 3-38: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola* 3 where red blot= T1(chemical SNPs), blue

plot= T2(biological SNPs), yellow plot=T3(imipenem) and green plot= control, red line = threshold line.

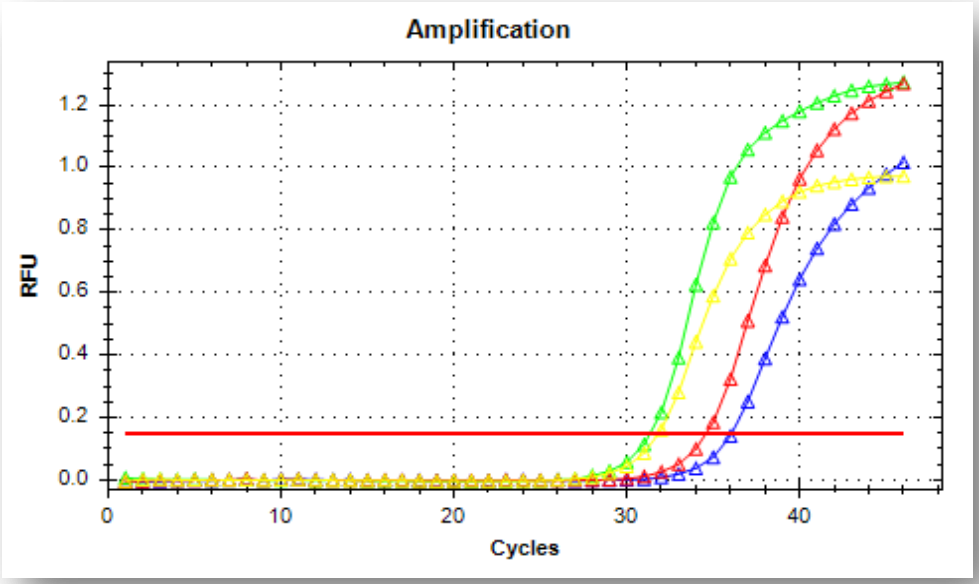


Figure 3-39: RT- qPCR amplification biofilm formation genes (*esaL*) in treated and untreated (control) *Pantoea* sp. 1,2 where red blot= T1(chemical SNPs), blue plot= T2(biological SNPs), yellow plot=T3(imipenem) green plot= control, red line = threshold line

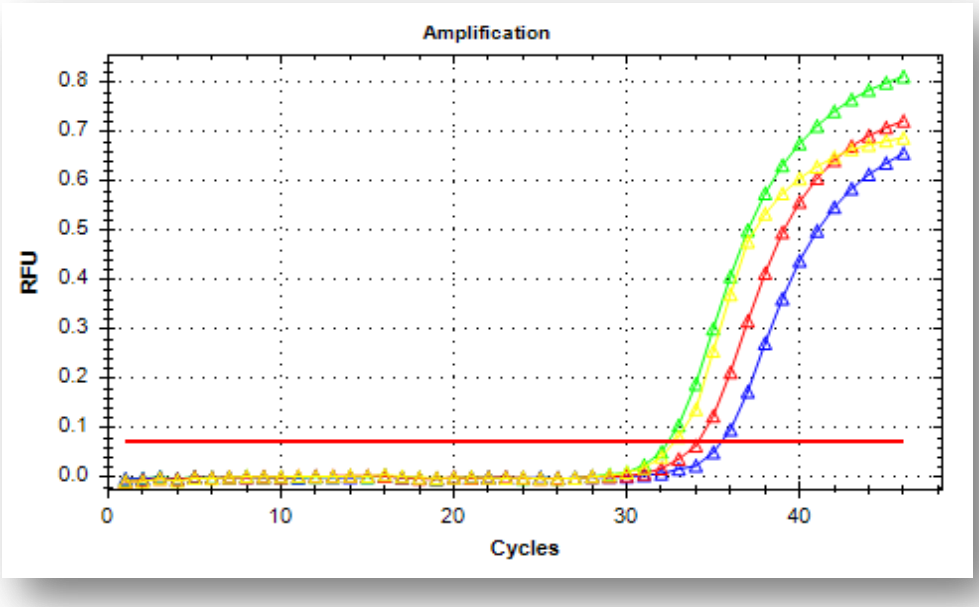


Figure 3-40: RT- qPCR amplification biofilm formation genes (*esaL*) in treated

and untreated (control) *Pantoea* sp. 3,4 where red blot= T1(chemical SNPs), blue plot= T2(biological SNPs), yellow plot=T3(imipenem), green plot= control, red line = threshold line

The results revealed that gene expression of biofilm genes *(icaA, smaI* and *esaL*) could be underexpressed when biofilm formation bacterial cell (*S. lentus*, *S. fonticola* and *Pantoea* sp.) treated with SNPs chemically, biologically, antibiotics (imipenem and azithromycin) and the combination between them may lead to loss its biofilm's ability. These expression was termed "RFU" which mean "relative fluorescence units" is a measurement unit used in RT-PCR analysis to detect fluorescence signal (Hellemans and Vandesompele, 2014).

The cycle number a cross red line is called the threshold cycle, or C_T which represent a cycle number of PCR that gene expression occur (Schmittgen and Livak, 2008). C_T , is a value PCR cycle at which the fluorescent signal determine the amplification to each gene by monitoring it to represent expression level (Fu *et al*., 2006).

Relative quantification gene expression was determined according to C_T value to test and housekeeping gene and differences between them to determine the fold change in gene expression (Fu *et al*., 2006) based on the ΔCT method reference gene equation, It was more simple in perform than livak method and give same results (Schmittgen and Livak, 2008).

Ratio (reference/target) = 2^ CT(reference) – CT(target) = fold change

Fold changes were represented by relative gene expression of target genes (*icaA, smaI* and *esaL*) that normalized to reference gene (*rpoB*). The using reference gene to normalize the target gene expression was very important to estimate the fold change in gene expression and

accurate results because is considered highly conserved expressed RNA subunit and used as constant standard (Liu *et al*., 2016).

To calculate fold change gene expression, 2up to differences between C_T test and housekeeping gene are shown in (Fig. 3-41, 3-42, 3-43, 3-44, 3-45) which represent the expression level of (*icaA, smaI* and *esal*) during biofilm formation. The level of *icaA, smaI* and *esal* expression decreased dramatically for the *S. lentus*, *S. fonticola* and *Pantoea* sp. isolates respectively were cultured with SNPs chemically and biologically and selected antibiotics (imipenem and azithromycin) (with varying influence) in compared to level of gene expression of control isolates (broth of biofilm bacteria with sugar and without SNPs or antibiotics treating).

These results may explain that SNPs in both types and antibiotics mechanism of action when down regulate gene expression of *smaI* and *esaL* genes may degrade the quarm sensing signal which regulate biofilm formation and effect on *icaA* gene inhibit intracellular adhesion which assist in biofilm formation. Some studies pointed that expression of *icaA* gene in staphylococci may modulated by many stress factors (Resch *et al*., 2005). In chines study, chitosan inhibit staphylococci biofilm formation by down-regulate *icaA* gene expression as result of treated with chitosan (Tan et al., 2012).

The fold change of *icaA* expression level was ranged from 0.406-2.137 in compared to 3.145 of control *S. lentus* isolate (Fig. 3-41).

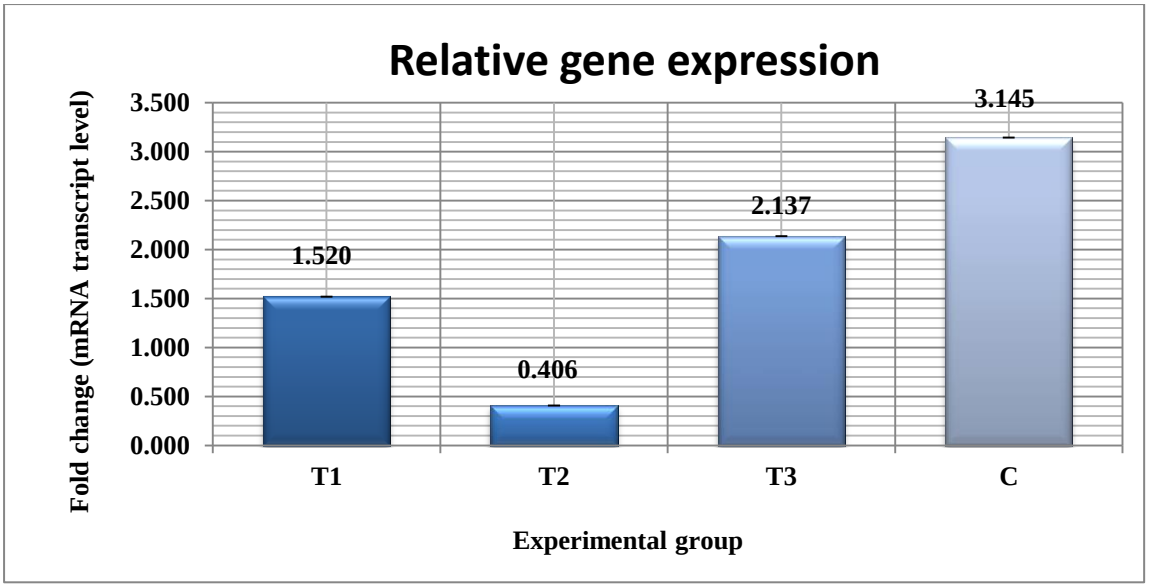


Figure 3-41: Mean of fold change in gene expression of *icaA* in biofilm *S. lentus* (T1= chemical SNPs, T2= biological SNPs, T3= azithromycin, C= control (*S. lentus* with broth +sugar)

While the fold change of *smaI* expression level was ranged from 1.640-3.678 in compared to 6.953 of control *S. fonticola* isolate 1,2 and ranged from 0.939- 3.015 to control *S. fonticola* isolate 3 (Fig. 3-42 and 3-43).

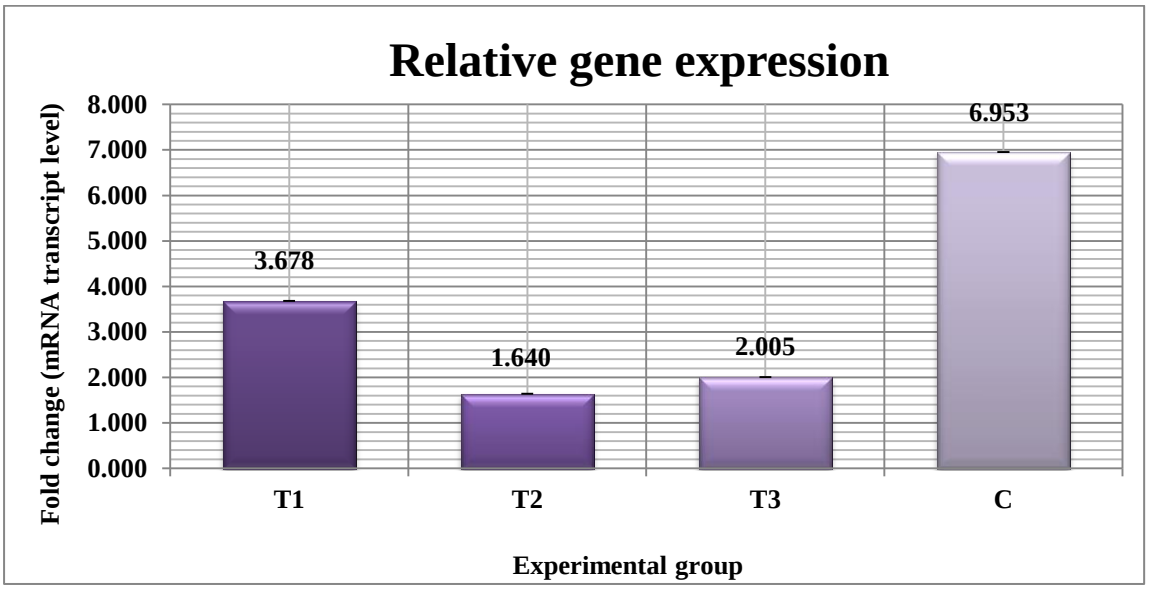


Figure 3-42: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola* 1,2 (T1= chemical SNPs, T2= biological SNPs, T3= imipenem, C= control (*S. fonticola* with broth +sugar)

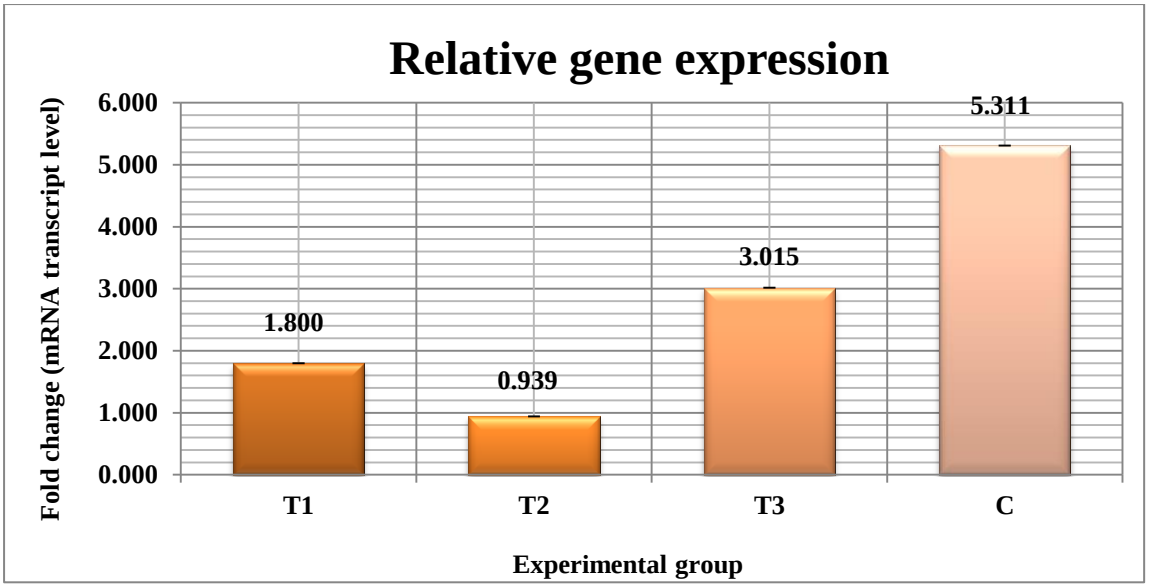


Figure 3-43: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola* 3 (T1= chemical SNPs, T2= biological SNPs, T3= imipenem, C= control (*S. fonticola* broth and sugar)

The fold change of *esaL* expression was ranged from 2.666- 4.468 in compared to 6.735 of control *Pantoea* sp. isolates 1,2 and ranged from 1.090- 3.089 in compared to 5.593 of control *Pantoea* sp. isolates 3,4 (Fig 3-44 and 3-45).

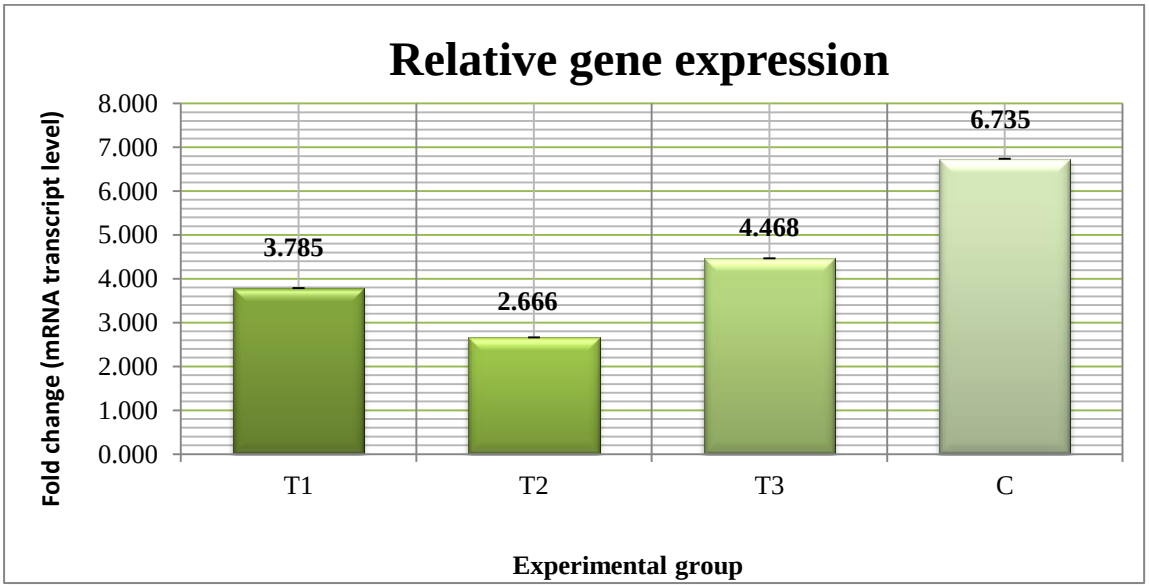


Figure 3-44: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea* sp.1,2 (T1= chemical SNPs, T2= biological SNPs, T3= imipenem, C= control (*Pantoea* sp. broth and sugar)

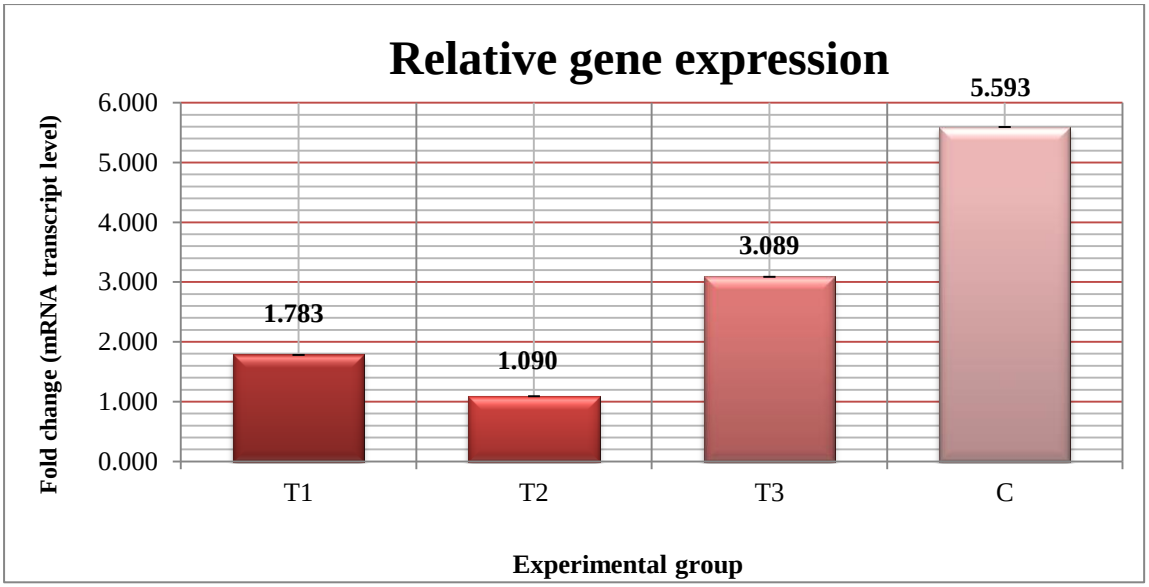


Figure 3-45: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea* sp.3,4 (T1= chemical SNPs, T2= biological SNPs, T3= imipenem, C= control (*Pantoea* sp. broth and sugar)

All figures show that biological SNPs were more influence on gene expression of tested biofilm bacterial genes than chemical SNPs or antibiotics with significant difference *P*= 0.002, 0.009, 0.001, 0.006 and 0.007 to biofilm bacterial isolates (*S. lentus*1,2,3, *S. fonticola* 1,2, *S. fonticola* 3, *Pantoea* sp. 1,2, *Pantoea* sp. 3,4) respectively (Appendix 5,6,7,8,9) and that genotypic effects was associated with phenotypic effects of biological SNPs on tested biofilm bacteria which revealed more potent antibacterial effect than others.

3.8.2.2. Combination between SNPs and antibiotics:

At genetic level the gene expression also influence with combination as well as phenotypic level as increase down-regulate of expression to tested genes compared to individual antimicrobial agents as shown in (Figs. 3-46, 3-47, 3-48, 3-49, 3-50, 3-51, 3-52, 3-53, 3-54, 3-55) for combination of chemical SNPs with antibiotics (AZM and IMP) and (Figs. 3-56, 3-57, 3-58, 3-59, 3-60, 3-61, 3-62, 3-63, 3-64, 3-65) for

combination of biological SNPS with antibiotics (AZM and IMP). The results revealed that gene expression of biofilm encoding genes (*icaA, smaI* and *esaL*) were decreased as a result of combination effect of chemical and biological SNPs with imipenem, and chemical and biological SNPs with azithromycin than each one alone. Each treatment was a macrodilution broth of mixing SNPs with antibiotic at below, equal and above MIC concentration.

The *icaA* expression level was decreased from 7.260 to 0.186 according to excess of combination chemical SNPs and azithromycin concentration in compared to control *S. lentus* isolates (Fig. 3-46 and 3-47).

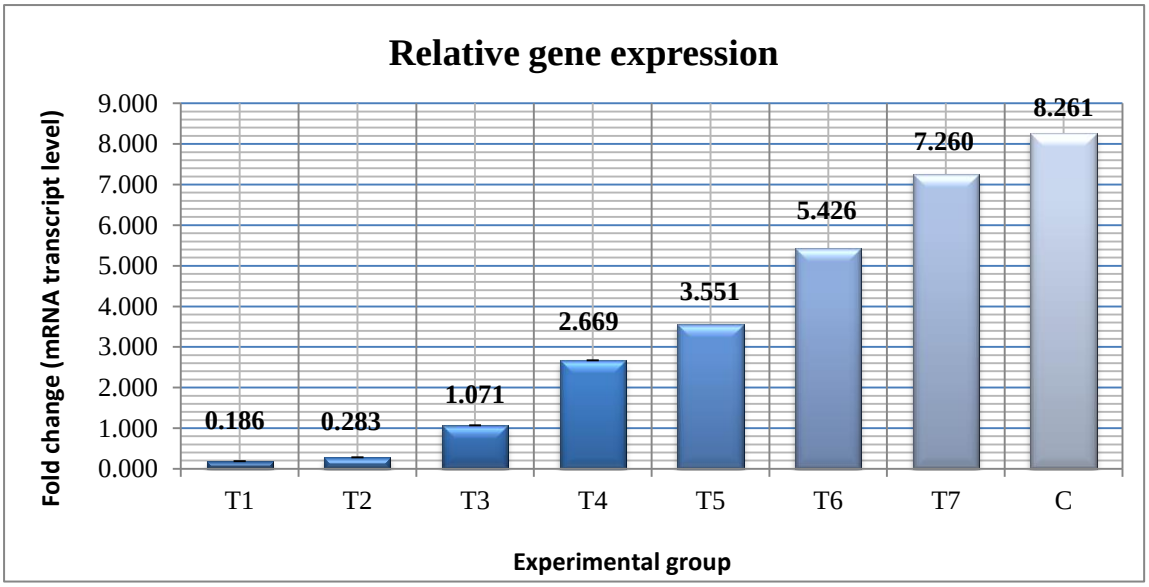


Figure 3-46: Mean of fold change in gene expression of *icaA* in biofilm *S. lentus* isolates treated with combination of chemical SNPs and azithromycin (T1=170/512, T2=150/256, T3=130/128, T4=110/64, T5=90/32, T6=70/16, T7=50/8, C= control (*S. lentus* broth and sugar)

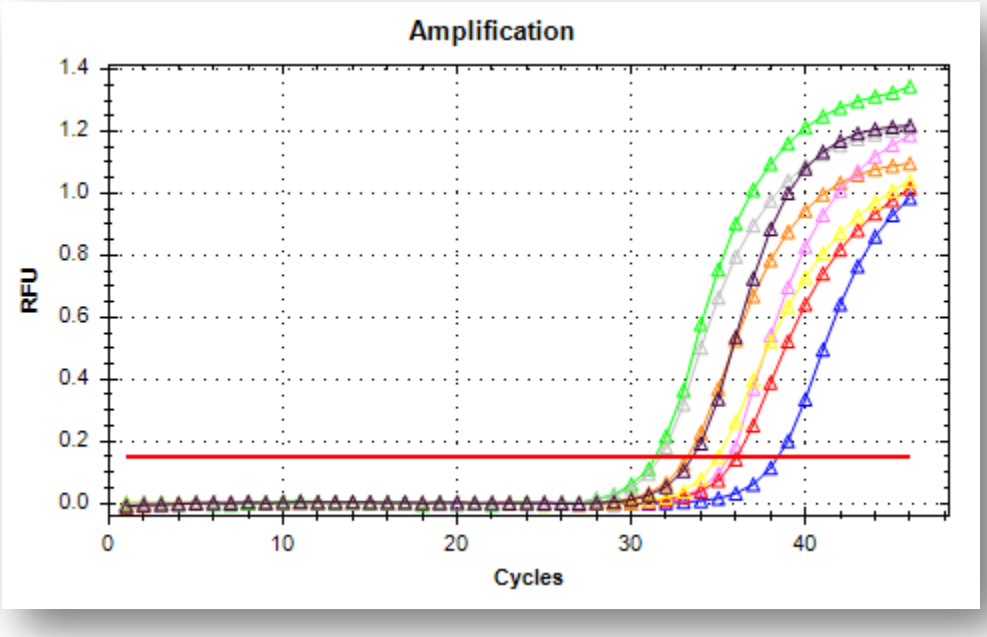


Figure 3-47: RT- qPCR amplification biofilm formation genes (*icaA*) in treated and untreated (control) *S. lentus* where red blot= T1, blue plot= T2, yellow plot=T3, orange plot=T4, black plot=T5, pink plot=T6, gray plot= T7 and green plot= control, red line = threshold line.

The *smaI* expression level was decreased from 7.417 to 0.455 according to excess of combination chemical SNPs and imipenem concentration in compared to control *S. fonticola* isolates1,2 (Fig. 3-48 and 3-49).

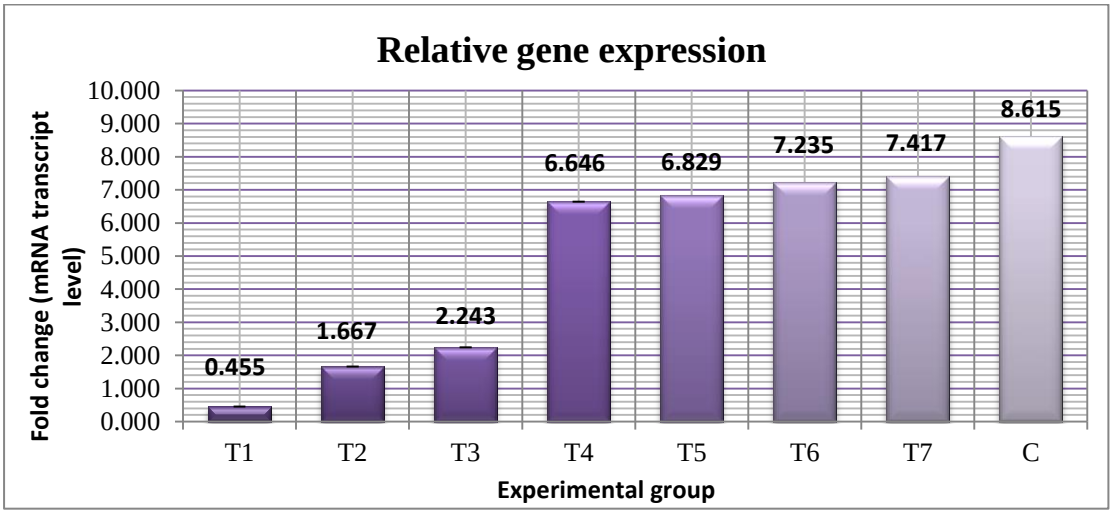


Figure 3-48: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola*1,2 treated with combination of chemical SNPs and imipenem (T1=170/8, T2=150/4, T3=130/2, T4=110/1, T5=90/0.5, T6=70/0.25, T7=50/0.125, C= control (*S. fonticola* broth and sugar)

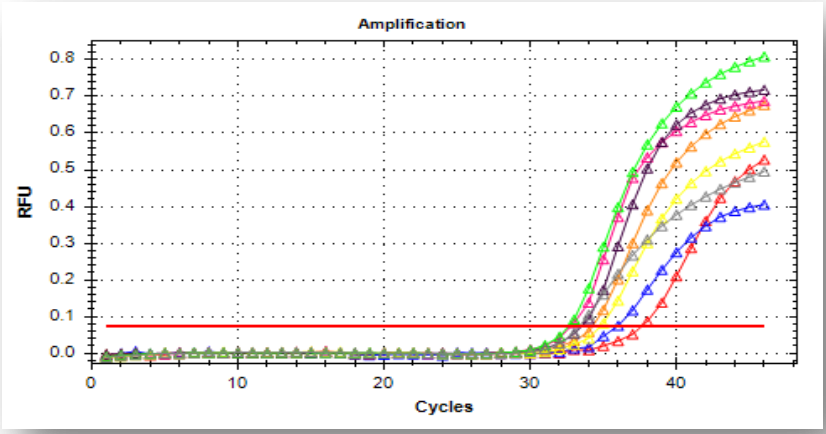


Figure 3-49: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola* 1 where red blot= T1, blue plot= T2, yellow plot=T3, orange plot=T4, black plot=T5, pink plot=T6, gray plot= T7 and green plot= control, red line = threshold line

The *smaI* expression level was decreased from 9.948 to 0.160 according to excess of combination chemical SNPs and imipenem concentration in compared to control *S. fonticola* isolate 3 (Fig. 3-50 and 3-51).

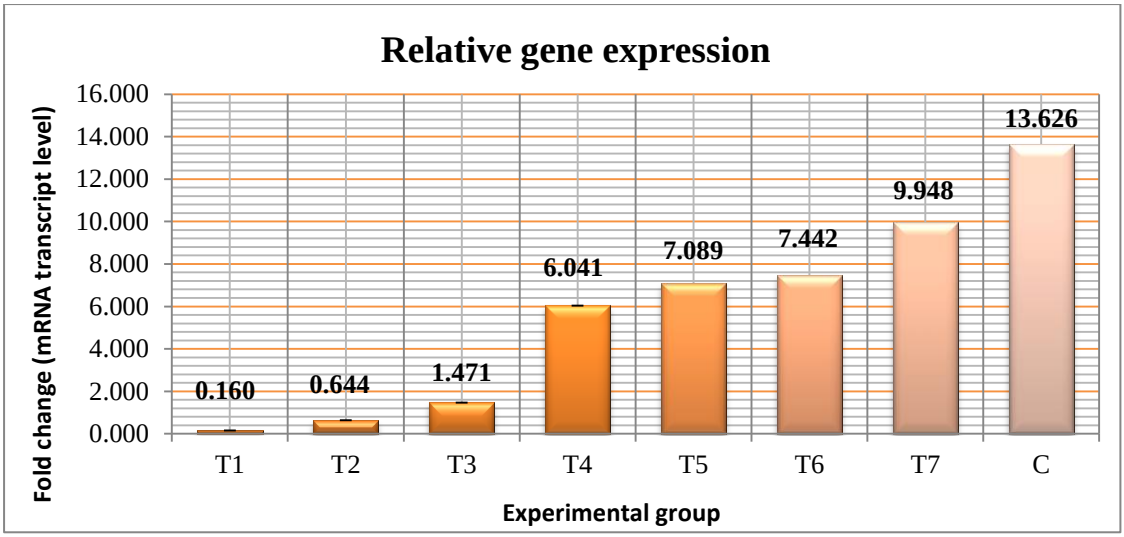


Figure 3-50: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola* 3 treated with combination of chemical SNPs and imipenem (T1=170/8, T2=150/4, T3=130/2, T4=110/1, T5=90/0.5, T6=70/0.25, T7=50/0.125, C= control (*S. fonticola* broth and sugar)

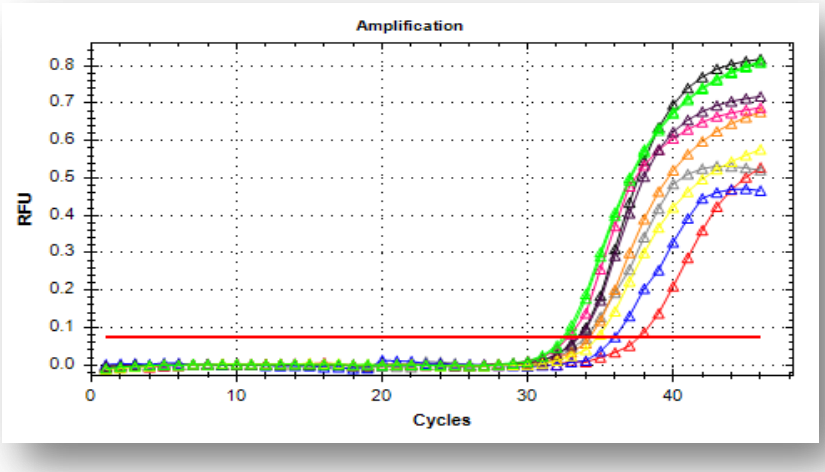


Figure 3-51: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola* 2 where red blot= T1, blue plot= T2, yellow plot=T3, orange plot=T4, black plot=T5, pink plot=T6, gray plot= T7 and green plot= control, red line = threshold line

The *esaL* expression level was decreased from 4.465 to 1.643 according to excess of combination chemical SNPs and imipenem

concentration in compared to control *Pantoea* sp. isolates1,2 (Fig. 3-52 and 3-53).

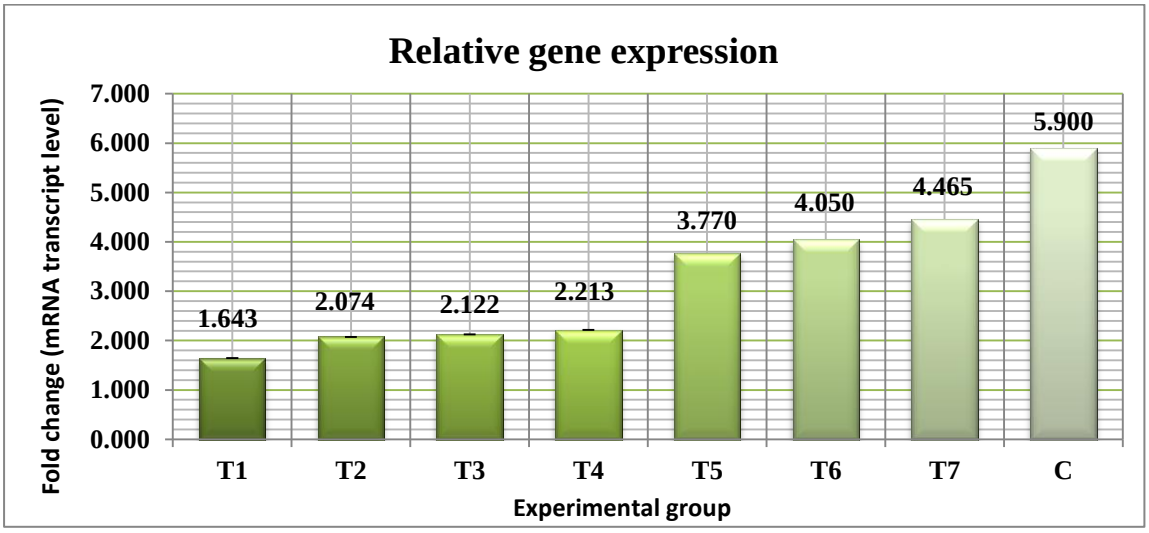


Figure 3-52: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea sp.*1, 2 treated with combination of chemical SNPs and imipenem (T1=170/8, T2=150/4, T3=130/2, T4=110/1, T5=90/0.5, T6=70/0.25, T7=50/0.125, C= control (*Pantoea* sp. broth and sugar)

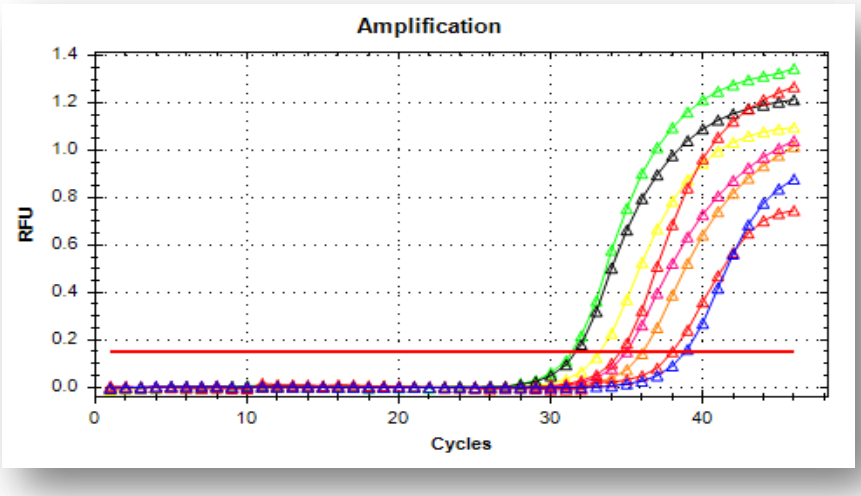


Figure 3-53: RT- qPCR amplification biofilm formation genes (*esaL*) in treated and untreated (control) *Pantoea* sp. where red blot= T1, blue plot= T2, yellow plot=T3, orange plot=T4, black plot=T5, pink plot=T6, gray plot= T7 and green plot= control, red line = threshold line

The *esaL* expression level was decreased from 5.126 to 0.583 according to excess of combination chemical SNPs and imipenem concentration in compared to control *Pantoea* sp. isolates 3,4 (Fig. 3-54 and 3-55).

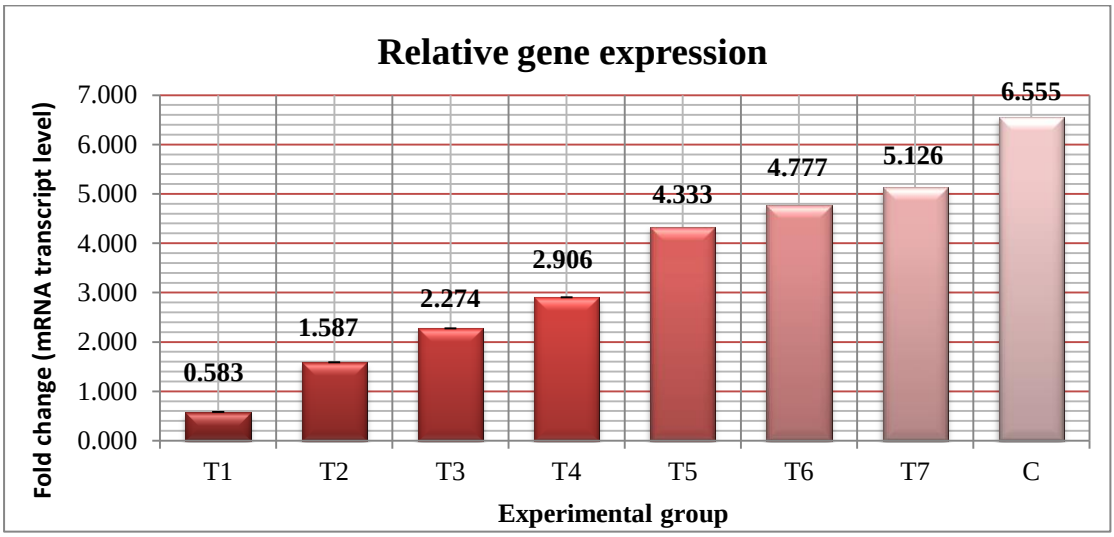


Figure 3-54: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea* sp. 3,4 treated with combination of chemical SNPs and imipenem (T1=170/8, T2=150/4, T3=130/2, T4=110/1, T5=90/0.5, T6=70/0.25, T7=50/0.125, C= control (*Pantoea* sp. broth and sugar)

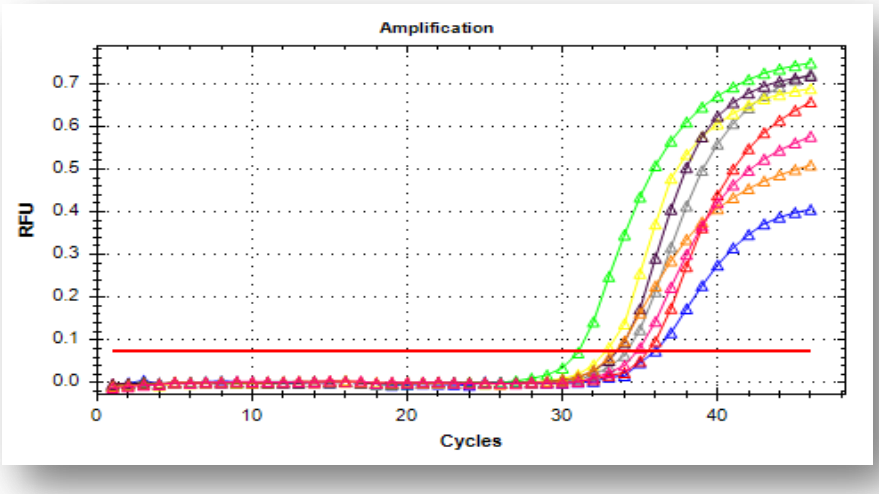


Figure 3-55: RT- qPCR amplification biofilm formation genes (*esaL*) in treated and untreated (control) *Pantoea* sp. where red blot= T1, blue plot= T2, yellow

plot=T3, orange plot=T4, black plot=T5, pink plot=T6, gray plot= T7 and green plot= control, red line = threshold line.

While in combination of biological SNPs with azithromycin, the *icaA* expression level was decreased from 5.340 to 1.065 according to excess of combination concentration in compared to control *S.lentus* isolates (Fig. 3-56 and 3-57).

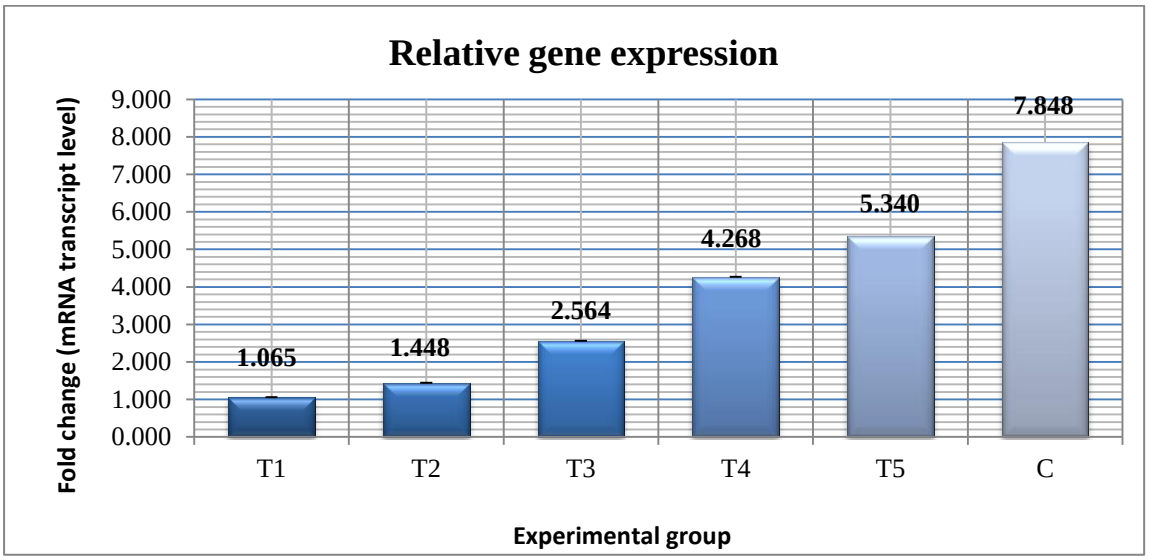


Figure 3-56: Mean of fold change in gene expression of *icaA* in biofilm *S. lentus* treated with combination of biological SNPs and azithromycin (T1=85/512, T2=65/256, T3=45/128, T4=25/64, T5=5/32, C= control (*S. lentus* broth and sugar)

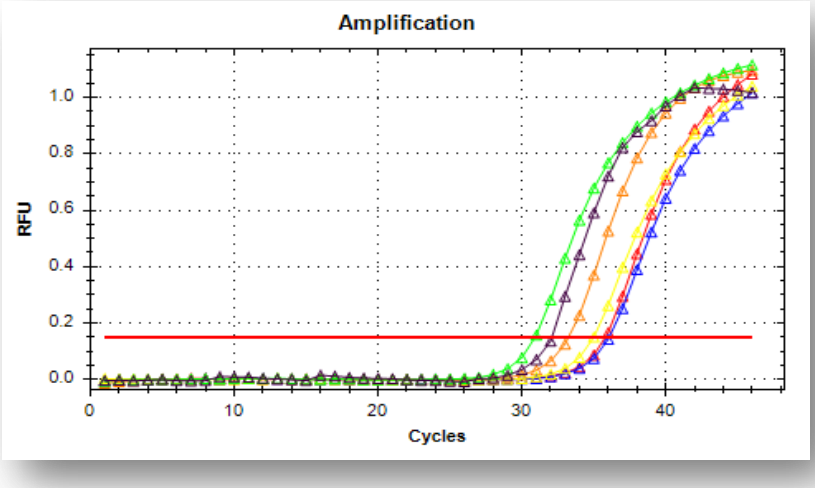


Figure 3-57: RT- qPCR amplification biofilm formation genes (*icaA*) in treated and untreated (control) *S. lentus* where red blot= T1, blue plot= T2, yellow plot=T3, orange plot= T4, black plot= T5 and green plot= control, red line = threshold line.

The *smaI* expression level was decreased from 6.208 to 1.029 according to excess of combination biological SNPs and imipenem concentration in compared to control *S. fonticola* isolates 1,2 (Fig. 3-58 and 3-59).

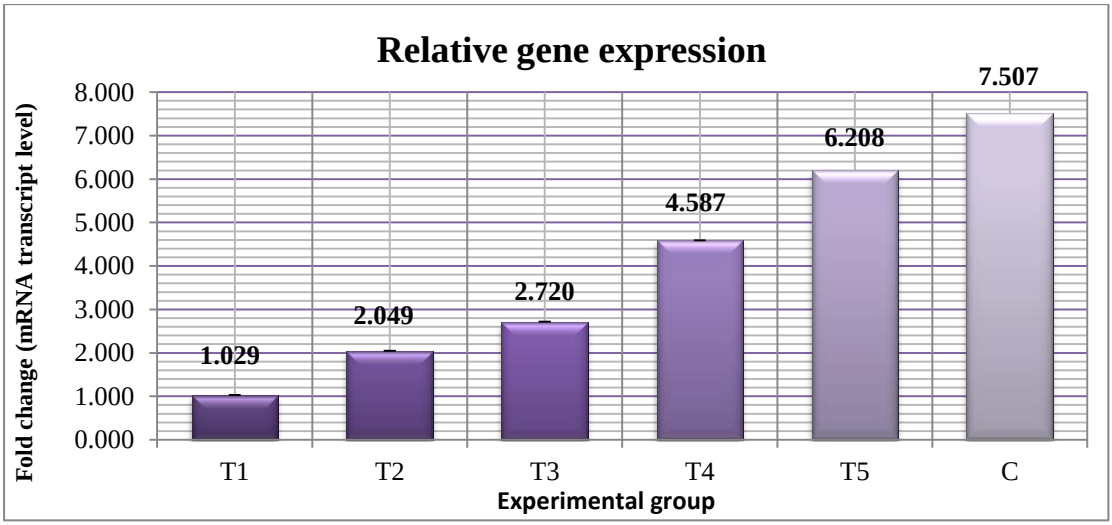


Figure 3-58: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola*1,2 treated with combination of biological SNPs and imipenem (T1=85/8, T2=65/4, T3=45/2, T4=25/1, T5=5/0.5, C= control (*S. fonticola* with broth and sugar)

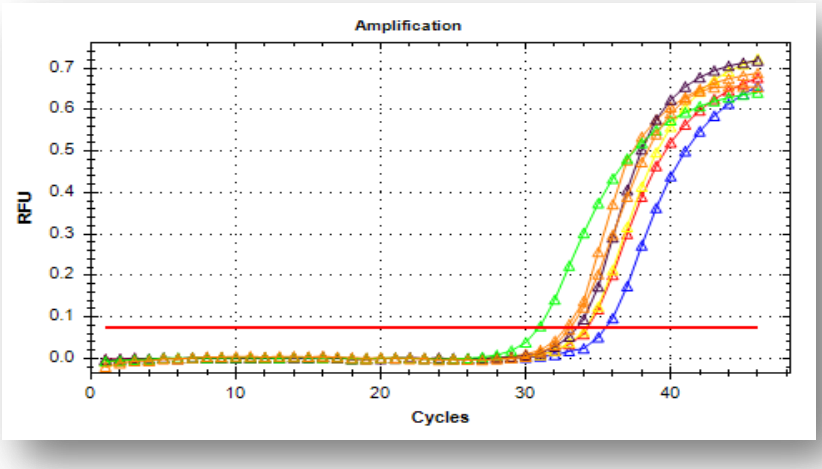


Figure 3-59: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola 1*. where red blot= T1, blue plot= T2, yellow plot=T3, orange plot= T4, black plot= T5 and green plot= control, red line = threshold line.

The *smaI* expression level was decreased from 6.657 to 0.234 according to excess of combination biological SNPs and imipenem concentration in compared to control *S. fonticola* isolates 3,4 (Fig. 3-60 and 3-61).

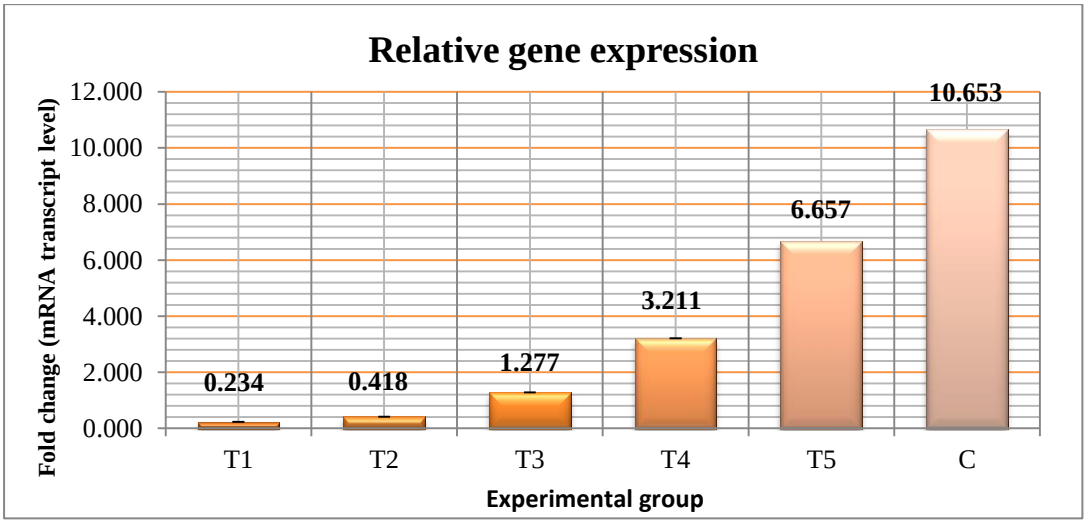


Figure 3-60: Mean of fold change in gene expression of *smaI* in biofilm *S. fonticola* 3 treated with combination of biological SNPs and imipenem (T1=85/8, T2=65/4, T3=45/2, T4=25/1, T5=5/0.5, C= control (*S. fonticola* broth and sugar)

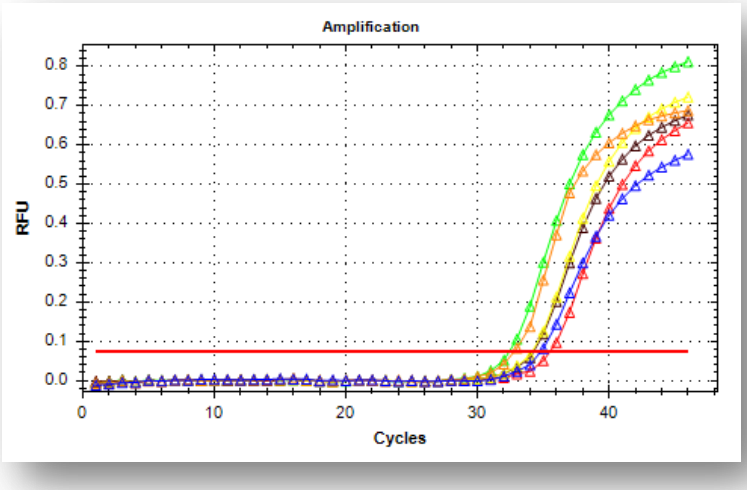


Figure 3-61: RT- qPCR amplification biofilm formation genes (*smaI*) in treated and untreated (control) *S. fonticola 2* where red blot= T1, blue plot= T2, yellow plot=T3, orange plot= T4, black plot= T5 and green plot= control, red line = threshold line.

The *esaL* expression level was decreased from 2.689 to 0.254 according to excess of combination biological SNPs and imipenem concentration in compared to control *Pantoea* sp. isolates 1,2 (Fig. 3-62 and 3-63).

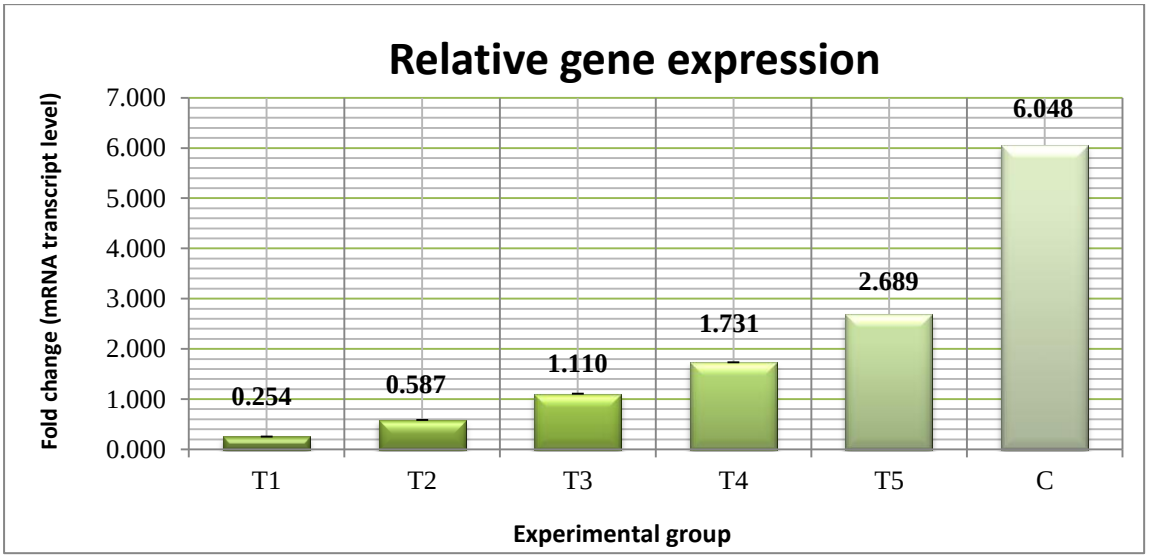


Figure 3-62: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea* sp. 1, 2 treated with combination of biological SNPs and imipenem (T1=85/8, T2=65/4, T3=45/2, T4=25/1, T5=5/0.5, C= control (*Pantoea* sp. with broth and sugar)

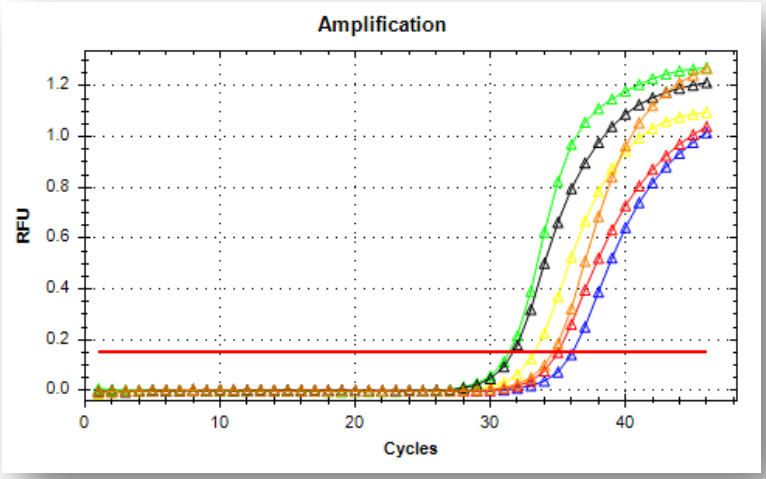


Figure 3-63: RT- qPCR amplification biofilm formation genes (*esaL*) in treated and untreated (control) *Pantoea* sp. where red blot= T1, blue plot= T2, yellow plot=T3, orange plot= T4, black plot= T5 and green plot= control, red line = threshold line.

The *esaL* expression level was decreased from 5.130 to 1.895 according to excess of combination biological SNPs and imipenem concentration in compared to control *Pantoea* sp. isolates 3,4 (Fig. 3-64 and 3-65).

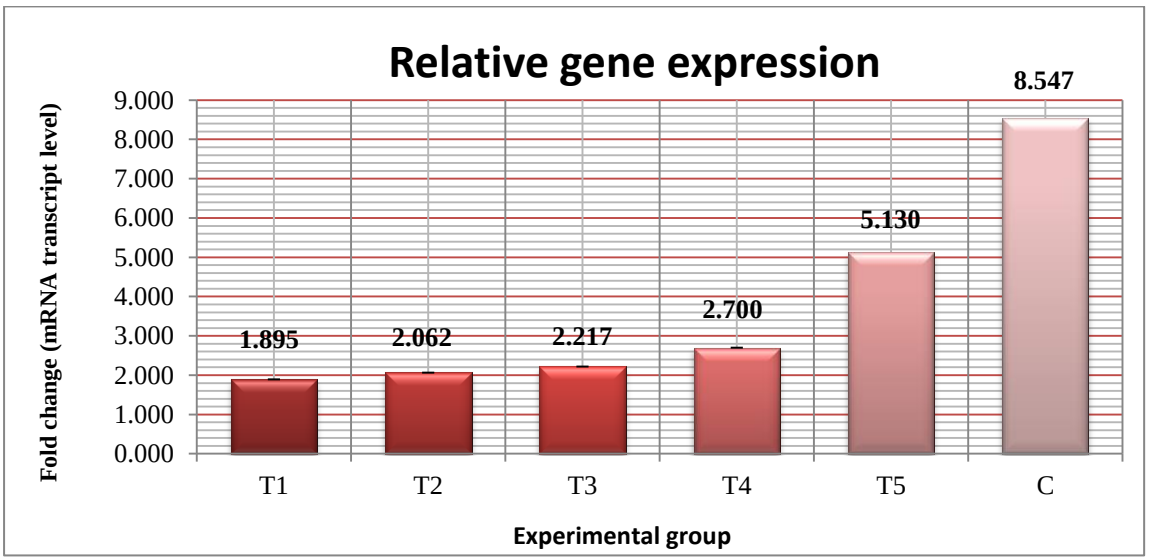


Figure 3-64: Mean of fold change in gene expression of *esaL* in biofilm *Pantoea* sp. 3,4 treated with combination of biological SNPs and imipenem (T1=85/8, T2=65/4, T3=45/2, T4=25/1, T5=5/0.5, C= control (*Pantoea* sp. with broth and sugar).

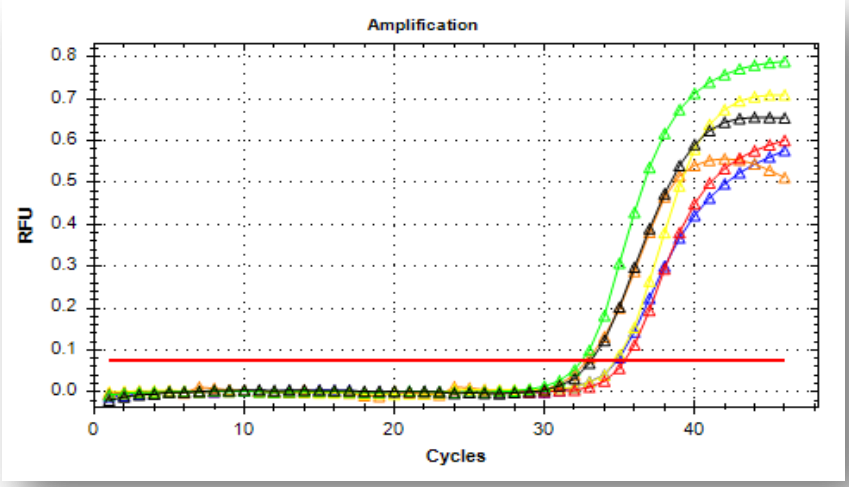


Figure 3-65: RT- qPCR amplification biofilm formation genes (*esaL*) in treated and untreated (control) *Pantoea* sp. where red blot= T1, blue plot= T2, yellow plot=T3, orange plot= T4, black plot= T5 and green plot= control, red line = threshold line.

Combination therapy potentially has many benefits compared to individual antibiotic therapy specifically in the cases of severe infections. Combinations would increase the effectiveness of the antimicrobial agents through synergism, increase the range of bacteria targeted in empirical therapy through affecting different targets, limit virulence factors expression, and prevent the development of antibiotic resistance (Wilkinson, 2016). Statistical analysis revealed that combination between chemical and biological SNPs with azithromycin on all *S. lentus* isolates significant difference ($p<0.001$, 0.00.4) respectively (Appendix 10,11). While the combination between chemical and biological SNPs with imipenem on *S. fonticola* 1,2 isolates revealed a significant difference p= 0.002 to chemical SNPs combination only (Appendix 12,13), but *S.*

fonticola 3 shows a significant differences p< 0.001 in chemical and biological SNPs combination (Appendix 14, 15). The combination effect of chemical and biological SNPs with imipenem on *Pantoea* sp. isolates revealed a significant difference p< 0.001 in combination of biological SNPs with imipenem only to *Pantoea* sp. 1, 2 (Appendix 16, 17, 18, 19) respectively. t test analysis in comparison between combination of chemical SNPs with antibiotics and biological SNPs with antibiotics showed that no significant difference p> 0.005 in all experimental biofilm bacterial isolates (Appendix 20, 21, 22, 23, 24) to *S. lentus*, *S. fonticola* 1,2, *S. fonticola* 3, *Pantoea* sp. 1,2, *Pantoea* sp. 3,4 respectively.

Conclusions and Recommendation

Conclusions :

1. Urinary catheter was the most important source of biofilm formation pathogens.
2. The major biofilm formation bacteria were belong to enterobactericeae family and *S. lentus* represented all gram positive isolates.
3. Locally, *S. lentus, S. fonticola* and *Pantoea* sp. were isolated for the first time from urine of catheterized patients as biofilm former
4. *Pantoea* sp. was a strong biofilm producer in addition to *Proteus mirabilis, E. coli and Yersinia enterocolitica*.
5. All selected biofilm forming bacteria display highly resistant rate to most tested antibiotics.
6. Only imipenem was affected biofilm bacterial growth (*S. fonticola* and *Pantoea* sp.) and azithromycin on *S. lentus*.
7. In comparative with chemical synthesized SNPs, biological SNPs were more potent antimicrobial effect on biofilm bacterial isolates.
8. Synergism effect of SNPs and antibiotics as a results of combination them effects on selected biofilm forming bacteria which enhance their antimicrobial activity.
9. Gene expression of biofilm encoding genes (*icaA, smaI* and *esaL*) were down regulate when treated with SNPs in both types and when treated with combination of SNPs and antibiotics, but most effective among them was biological SNPs alone and in combination with antibiotics.

Recommendations:

- Urine sample should be routinely screened for biofilm formation.
- Urinary catheter should be inserted under completely aseptic technique and should be removed as early as possible
- The toxicity of SNPs should be evaluated before developing them for clinical applications.
- Further study of antimicrobial effect of SNPs in vivo by using animals lab is needed.
- Extensive studies about antibiotic resistant gene in biofilm forming bacteria is needed.

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Appendices

Appendix 1: A questionnaire form was used in present study:

Sample no. : date:

Name : age: sex:

Antibiotics : address:

History:

Disease:

Other notes:

Diagnosis:

Appendix 2: Statistical analysis of Optical density value of bacterial biofilm degree according to TCP method:

Biofilm bacteria	Optical density
S. lentus	0.1736±0.0368 A
P. mirabilis	0.4188±0.1618 B
K. pneumonia	0.1962±0.0526 A
S. fonticola	0.2520±0.0450 A
Pantoea sp.	0.6597±0.1462 B
E. coli	0.2410±0.1160 A
P. oryzihabitance	0.2150±0.1500 A
E. aerogenes	0.1380±0.0130 A
E. cloacae	0.3540±0.0012 B
Y. enterocolitica	0.6790±0.0111 B

Values represent mean ±S.E

Different capital letters mean significant differences (P≤0.05) between different optical densities

Appendix 3: Statistical analysis of susceptibility of *Pantoea sp.* isolates and control to imipenem according to inhibition zone.

Bacteria	Inhibition zone +SE
Pantoea sp. 1	35± 0.577 A
Pantoea sp. 2	15±0.577 B
Pantoea sp. 3	34±0.577 A
Pantoea sp. 4	33±0.577 C
Control *E. coli*	39±5.77 D

Note: values represent mean ±S.E

Different capital letters mean significant differences (P≤0.05) between different inhibition zones.

Appendix 4: Statistical analysis of susceptibility of *S. fonticola* isolates and control to imipenem according to inhibition zone.

Bacteria	Inhibition zone ±SE
S. fonticola1	38±0.577 A
S. fonticola2	36±1.15 A
S. fonticola3	35±1.15 A
S. mercenes	40±1.15 B

Note: values represent mean ±S.E

Different capital letters mean significant differences (P≤0.05) between different inhibition zones.

Appendix 5: Statistical analysis of gene expression of *S. lentus* isolates after treatment with chemical and biological SNPs and azithromycin.

Sample	Mean ± SE	P-value
T1	1.520±0.156	0.002
T2	0.406±0.052	
T3	2.137±0.566	
T4	3.144±0.357	

Appendix 6:Statistical analysis of gene expression of *S. fonticola* 1,2 isolates after treatment with chemical and biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	3.6787±0.22704	0.009
T2	1.6407±0.36673	
T3	2.0047±0.02598	
T4	6.9533±1.65687	

Appendix 7: Statistical analysis of gene expression of S. fonticola 3 isolates after treatment with chemical and biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	1.8003±0.47420	0.001
T2	.9387±0.20402	
T3	3.0150±0.31802	
T4	5.3103±0.71751	

Appendix 8: Statistical analysis of gene expression of Pantoea sp.1,2 isolates after treatment chemical and biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	3.7857±0.93741	0.006
T2	2.6660±0.08166	
T3	4.4687±0.35743	
T4	6.7347±0.52331	

Appendix 9: Statistical analysis of gene expression of *Pantoea* sp.2,3 isolates after treatment chemical and biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	1.7827±0.04541	0.007
T2	1.0897±0.09381	
T3	3.0890±1.13238	
T4	5.5933±0.73031	

Appendix 10: Statistical analysis of gene expression of *S. lentus* 1,2,3 isolates after treatment with combination of chemical SNPs and azithromycin.

Sample	Mean ± SE	P-value
T1	0.186±0.017	0.000
T2	0.283±0.395	
T3	1.071±0.148	
T4	2.668±0.668	
T5	3.550±1.349	
T6	5.426± 1.124	

Appendix 11: Statistical analysis of gene expression of *S. lentus* 1,2,3 isolates after treatment with combination of biological SNPs and azithromycin

Sample	Mean ± SE	P-value
T1	1.065±0.102	0.004
T2	1.448±0.105	
T3	2.564±0.652	
T4	4.268±0.791	
T5	5.339±1.752	
Control	7.848±1.475	

Appendix 12: Statistical analysis of gene expression of *S. fonticola* 1,2 isolates after treatment with combination of chemical SNPs and imipenem

Sample	Mean ± SE	P-value
T1	0.4547±0.08673	0.002
T2	1.6673±0.76251	
T3	2.2427±0.13626	
T4	6.6463±0.84380	
T5	6.8287±1.87678	
T6	7.2350±1.42994	
T7	7.4167±0.94686	
Control	8.6147±2.51172	

Appendix 13: Statistical analysis of gene expression of *S. fonticola* 1,2 isolates after treatment with combination of biological SNPs and imipenem

Sample	Mean ± SE	P-value
T1	1.0293±0.03656	0.244
T2	2.0493±0.69233	
T3	2.7200±0.87973	
T4	4.5873±0.25321	
T5	6.2080±4.62522	
Control	7.5070±1.30800	

Appendix 14: Statistical analysis of gene expression of *S. fonticola* 3 after treatment with combination of chemical SNPs and imipenem

Sample	Mean ± SE	P-value
T1	0.1600±0.12879	0.000
T2	0.6437±0.05795	
T3	1.4707±0.15057	
T4	6.0410±0.75446	
T5	7.0890±2.56477	
T6	7.4423±2.24652	
T7	9.9467±1.76125	
Control	13.6263±1.22144	

Appendix 15: Statistical analysis of gene expression of *S. fonticola* 3 after treatment with combination of biological SNPs and imipenem

Sample	Mean ± SE	P-value
T1	0.2340±0.05186	0.000
T2	0.4177±0.14389	
T3	1.2767±0.13135	
T4	3.2110±0.64451	
T5	6.6567±1.84217	

Control	10.6527±2.37459	

Appendix 16: Statistical analysis of gene expression of *Pantoea* sp.1,2 after treatment with combination of chemical SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	1.6430±0.76022	0.062
T2	2.0737±0.07872	
T3	2.1220±0.70909	
T4	2.2130±0.47422	
T5	3.7700±0.99895	
T6	4.0500±0.22398	
T7	4.4667±1.88540	
Control	5.8997±1.13478	

Appendix 17: Statistical analysis of gene expression of *Pantoea* sp.1,2 after treatment with combination of biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	0.2540±0.04013	0.000
T2	0.5867±0.15975	
T3	1.1097±0.08950	
T4	1.7313±0.23242	
T5	2.6893±0.73479	
Control	6.0473±0.89507	

Appendix 18: Statistical analysis of gene expression of *Pantoea* sp.3,4 after treatment with combination of chemical SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	0.5827±0.07670	0.006
T2	1.5870± 0.71255	
T3	2.2740±0.61451	
T4	2.9060±1.18727	
T5	4.3330±1.44956	
T6	4.7773±1.07099	
T7	5.1233±0.96446	
Control	6.5550±0.79581	

Appendix 19: Statistical analysis of gene expression of *Pantoea* sp.3,4 after treatment with combination of biological SNPs and imipenem.

Sample	Mean ± SE	P-value
T1	1.8947±0.11299	0.020
T2	2.0613±0.72306	
T3	2.2170±0.36003	
T4	2.7003±0.90174	
T5	5.1297±0.31823	
Control	8.5473±2.89352	

Appendix 20: T test analysis in comparison between combination of chemical and biological SNPs with antibiotics in *S. lentus* isolates.

Sample	Mean ± SE	P-value
Chemical	3.588± 0.666	0.861
biological	3.755± 0.672	

Appendix 21: T test analysis in comparison between combination of chemical and biological SNPs with antibiotics in s. fonticola 1,2 isolates.

Sample	Mean ± SE	P-value
Chemical	5.1383±0.72740	0.363
Biological	4.0168±0.88910	

Appendix 22: T test analysis in comparison between combination of chemical and biological SNPs with antibiotics in *S. fonticola* 3 isolate.

Sample	Mean ± SE	P-value
Chemical	5.1383±0.72740	0.363
Biological	4.0168±0.88910	

Appendix 23: T test analysis in comparison between combination of chemical and biological SNPs with antibiotics in *Pantoea* sp. 1,2 isolates.

Sample	Mean ± SE	P-value
Chemical	3.2798±0.40461	0.69
biological	2.0697±0.50096	

Appendix 24: T test analysis in comparison between combination of chemical and biological SNPs with antibiotics in *Pantoea* sp. 3,4 isolates.

Sample	Mean ± SE	P-value
Chemical	3.5173± 0.48084	0.785
biological	3.7584± 0.73142	

Appendix 25: Total RNA extraction of test and control of biofilm bacterial isolates with different treatments (cont.)

Treatment type	**Con. (ng/uL)**	**Purity 260/280nm**
Combination chemical SNPs and IMP	*Serratia fonticola* 1	
170/8	74.85	2.06
150/4	75.73	2.04
130/2	45.23	2.14
110/1	77.49	2.04
90/0.5	78.37	2.80
70/0.25	43.12	2.84
50/0.125	51.32	2.02
	S. fonticola 2	
170/8	43.21	2.40
150/4	61.32	2.06
130/2	52.65	2.13
110/1	42.22	2.12
90/0.5	51.76	2.23
70/0.25	65.32	2.29
50/0.125	54.23	2.09
	Pantoea sp. 1	
170/8	43.12	2.34
150/4	22.54	2.24
130/2	72.12	2.21
110/1	43.21	2.52
90/0.5	72.12	2.11
70/0.25	39.28	2.53
50/0.125	53.23	2.51
	Pantoea sp. 2	
170/8	49.02	2.39
150/4	61.65	2.29
130/2	45.12	2.34
110/1	45.22	2.12
90/0.5	65.34	2.12
70/0.25	54.23	2.01
50/0.125	103.87	2.14
	Pantoea sp. 3	
170/8	45.22	2.32
150/4	72.12	2.61
130/2	53.23	2.31

110/1	22.54	2.44
90/0.5	61.65	2.29
70/0.25	49.02	2.59
50/0.125	45.22	2.12
	Pantoea sp. 4	
170/8	54.23	2.01
150/4	39.28	2.03
130/2	53.23	2.11
110/1	61.65	2.09
90/0.5	45.12	2.34
70/0.25	65.34	2.12
50/0.125	54.23	2.01
	S. lentus 1	
170/8	49.02	2.59
150/4	61.65	2.49
130/2	45.12	2.24
110/1	45.22	2.32
90/0.5	65.34	2.12
70/0.25	54.23	2.01
50/0.125	103.87	2.54
	S. lentus 2	
170/8	43.12	2.64
150/4	22.54	2.44
130/2	72.12	2.41
110/1	43.21	2.52
90/0.5	72.12	2.21
70/0.25	39.28	2.33
50/0.125	53.23	2.21
	S. lentus 3	
170/8	49.02	2.19
150/4	61.65	2.39
130/2	45.12	2.34
110/1	45.22	2.02
90/0.5	65.34	2.12
70/0.25	54.23	2.01
50/0.125	103.87	2.44

Arabic Summary

الخلاصة:

تعد دقائق الفضة النانوية في الوقت الحالي مضادا حيويا فعالا خصوصا بعد ازدياد حالات الامراض المرافقة بتكون الغشاء الحيوي والمرتبطة مع مقاومة البكتريا للمضادات الحياتية لذلك دعت الضرورة لإيجاد طرق بديلة وجديدة لحل هذه المشكلة، لذلك هدفت هذه الدراسة الى تصنيع دقائق الفضة حيويا واستخدامها للقضاء على مسببات تكوين الغشاء الحيوي البكتيرية على المستوى المظهري والجيني ومقارنتها بتأثير دقائق الفضة المصنعة كيمياويا.

اجريت هذه الدراسة في الفترة من شباط ٢٠١٧ الى كانون الثاني ٢٠١٨ حيث جمعت خلالها ٦٥ عينة ادرار من مرضى مستخدمين لأنابيب القسطرة البولية الراقدين في مستشفيات الديوانية العام ومستشفى الحلة التعليمي ومستشفى الهاشمية والقاسم العام. حيث اظهرت النتائج ان٥٨ (٨٩,٢%) من عينات الادرار المفحوصة والمزروعة في المختبر تحتوي على عزلات بكتيرية وان ٢٨ (٤٣%) منها مكون للغشاء الحيوي بعد فحص قابليتها على تكوين الغشاء الحيوي من خلال فحص الكونغو ريد وفحص tissue culture plate وجينيا عن طريق تفاعل البوليمر المتسلسل لجينات مشفرة للغشاء الحيوي (*iacA, smaI* and *esaL*) الخاصة بالبكتريا المكونة للغشاء الحيوي المختارة (*Staphylococcus lentus, Serratia fonticola* and *Pantoea* sp.) حيث اظهرت جميعها قابليتها على انتاج الغشاء الحيوي.

شخصت العزلات البكتيرية المكونة للغشاء الحيوي بواسطة جهاز الفايتك حيث تصدرت بكتريا *Proteus mirabilis* بواقع ٥ عزلات تتبعها بكتريا *Klebsiella pneumoniae* و *Pantoea* sp. ب ٤ عزلات ثم *Pseudomonas oryzihabitans* و *Serratia fonticola* ب ٣ عزلات وعزلتين لكل من *Enterobacter aeruginosa* و *E. coli* وعزلة واحدة لكل من *Enterobacter cloacae* و *Yersinia enterocolitica* فيما تضمنت العزلات البكتيرية الموجبة لصبغة غرام على *Staphylococcus lentus* فقط ب ٣ عزلات.

تم فحص حساسية البكتريا المكونة للغشاء الحيوي للمضادات الحياتية للاجناس البكتيرية التالية (*Staphylococcus lentus, Serratia fonticola* and *Pantoea* sp)

عن طريق فحص الانتشار بالأقراص وفحص التركيز المثبط الادنى وفحص الحساسية بجهاز الفايتك حيث اظهرت النتائج ان البكتيريا المذكورة اعلاه مقاومة لأغلب المضادات المستخدمة في الدراسة ماعدا مضادي الازيثرومايسين الذي تحسست منه بكتريا *Staphylococcus lentus*بنسبة ١٠٠% ومضاد الاميبينيم الذي تحسست منه *Serratia fonticola* and *Pantoea* sp. بنسبة ١٠٠%

تم تصنيع دقائق الفضة النانوية باستخدام المعلق الخالي من الخلايا لبكتريا *Enterobacter cloacae* كعامل مختزل فيما استخدم الصوديوم بوروهايدرد كعامل مختزل في تصنيعها كيمياويا. قيمت هذه الدقائق المصنعة بكلا الطريقتين بعدة فحوص منها فحص تغاير اللون من عديم اللون او الاصفر الى اللون البني او البني الغامق بعد تحولها الى دقائق نانوية وقياس درجة الامتصاصية لجهاز المطياف الضوئي للأشعة فوق البنفسجية حيث سجلت دقاق الفضة المصنعة حيويا اعلى درجة امتصاص عند الطول الموجي ٤٠٠ نانوميتر وكيمياويا عند ٣٩٠ نانوميتر وكذلك تم قياس وجود مجموعة الكاربوكسيل والفينولك التي تغطي سطح الدقائق وتدعم ثباتية الجسيمات المصنعة وذلك باستخدام التحليل الطيفي للاشعة تحت الحمراء، كما تم استخدام المجهر الالكتروني الماسح وجهاز قياس حجم الجسيمات واظهروا ان حجم الدقائق المصنعة حيويا هو ٦٣ نانوميتر والمصنعة كيمياويا ٢٥ نانوميتر بشكل مكعبات مفردة.

كما تم دراسة جزيئات الفضة المصنعة كمضاد جرثومي حيث تم قياس تأثيرها على النمو البكتيري بواسطة طريقتين، طريقة الانتشار بالحفر وطريقة التخافيف بالأنابيب لقياس التركيز المثبط الادنى حيث اظهرت النتائج ان الدقائق النانوية المصنعة حيويا اكثر تأثيرا على نمو البكتريا المكونة للغشاء الحيوي المختارة (*Staphylococcus lentus, Serratia fonticola* and *Pantoea* sp.) من الدقائق المصنعة كيمياويا كذلك اظهرت النتائج ان تأثير الدقائق كمضاد حيوي يزداد بعد مزجها مع المضادات الحيوية (azithromycin and imipenem) ويكون اكثر فاعلية على نمو البكتريا اعلاه ومقارنتها بعزلات سيطرة ليظهر تأثيراً تأزرياً يثبت ان تأثير دقائق الفضة النانوية المضاد للجراثيم يتحسن بوجود المضادات الحيوية المذكورة.

قُيّم التعبير الجيني للجينات المشفرة لتكوين الغشاء الحيوي (*iacA, smaI* and *esaL*) عن طريق تفاعل البلمرة المتسلسل الكمي (RT qPCR) قبل وبعد معاملة البكتريا المكونة

للغشاء الحيوي بجزيئات الفضة النانوية المصنعة حيويا وكيمياويا ومعاملتها بالمضادات الحياتية وكذلك معاملتها بمزيج جزيئات الفضة مع المضادات الحياتية لكلا النوعين، حيث اظهرت نتائج التحليل ان التعبير الجيني للجينات المذكورة قلّ بعد معاملتها بجزيئات الفضة المصنعة حيويا وحدها او بعد مزجها بالمضادات الحياتية اكثر من تأثرها بالجزيئات المصنعة كيمياويا والمضادات الحياتية كل على حدة او بعد مزجها معا.

وزارة التعليم العالي و البحث العلمي
جامعة القادسية
كلية الطب
فرع الاحياء المجهرية

تأثير دقائق الفضة النانوية المحضرة حيويا وكيمياويا على البكتيريا المكونة للغشاء الحيوي والمعزولة من مرضى عراقيين مقسطرين بولياً

اطروحة مقدمة
الى مجلس كلية الطب/جامعة القادسية
و هي جزء من متطلبات نيل شهادة دكتوراه فلسفة في علم الاحياء المجهرية الطبية

من قبل

شيماء عبيد حسون
بكالوريوس علوم في علوم الحياة ـ ٢٠٠٢
ماجستير احياء مجهرية ـ ٢٠٠٧

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