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# Inhibitory effect of Ag/AgCl nanoparticles produced by *Lactobacillus* spp. against pathogenic bacteria

**A Thesis Submitted to the Council of College of sciences, University of Diyala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology**

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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We certify that this thesis entitled **" Inhibitory effect of Ag/AgCl nanoparticles produced by *Lactobacillus* spp. against pathogenic bacteria "** has been conducted under our supervision in partial fulfillment of the requirements for the degree of MSC in Biology Sciences at the College of sciences, University of Diyala.

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In view of the available recommendations I forward this thesis for debate

By the Examining Committee.

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I certify that the thesis entitled **Inhibitory effect of Ag/AgCl nanoparticles produced by *Lactobacillus* spp. against pathogenic bacteria** presented by **(Abdullah Saad Saab)** has been evaluated scientifically, therefore, it is suitable for debate by examining committee.

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# *Dedication*

To the candles that light up in the paths of my life

My Father ❤️

My Mother ❤️

&

My Family 👨‍👩‍👧 ❤️

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# Summary

From November 2019 to January 2020, two hundred Clinical specimens (Wound, Burns swabs and Urine samples) were collected from patients admitted to Baquba Teaching Hospital and Al-Batoul Hospital. Samples were cultured on MacConkey and Blood agar media. The bacterial isolates were then initially diagnosed using selective and differential media. Then biochemical tests were performed to confirm the diagnose of bacterial isolates. Based on the biochemical identification the bacterial spp. were as followings: *Klebsiella pneumoniae*, *Escherichia coli, Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and Staphylococcus aureus.

Antimicrobial susceptibility test, for all isolated bacterial species were evaluated toward 29 antimicrobial agents using disk diffusion method. The results showed that many bacterial isolates were multiple drugs resist (MDR).

Biosynthesis method of nanoparticles acquires very important area due to their economic and ecofriendly benefits. In the present study, used cell free supernatant of *lactobacillus* spp. for synthesis of silver nanoparticles. *Lactobacillus* spp. bacteria were isolated from local fermented product and identified as *Lactobacillus* spp. by using selective enrichment culture medium de Man, Rogosa and Sharpe agar (MRS agar). Morphology and microscopical diagnose and biochemical tests were used to confirm the identification of *Lactobacillus* . The cell free supernatants was used for biosynthesis of Silver nanoparticales.

The silver nanoparticles were Characterized using X-ray diffraction (XRD) analysis to confirm that the nanoparticles were silver and silver chloride

(AgCl) cubic types. The (crystalline) domains equation, showed three strongest peaks, the calculated particle size were 17.1and 18.6 nm for Ag, and 24.3 nm for AgCl. Atomic Force Microscopy (AFM) used to characterize the size, topography and granularity volume distribution of biosynthesized nanoparticles Ag/AgCl, average size was 50 nm. UV-visible spectroscopy revealed the formation of AgNPs. Fourier transform infrared (FTIR) spectroscopy showed different functional groups of biomolecules which were responsible for reduction and capping process. Electron microscopic (SEM) was used to characterize the shape and size of the synthesized nanoparticles.

The antibacterial activity of silver and sliver chloride nanoparticles against the selected multiple drugs resist (MDR) bacteria were determined by agar well diffusion method. It was observed that the growth of these bacteria was inhibited at 12,500 µg /ml of Ag&AgCl NPs. The nanoparticles concentrations were (12,500 ; 25,000 ; 50,000 ; 100,000) µg/ml had maximum inhibitory effect against *S. aureus* (24, 28, 30, 32 mm) respectively and minimum inhibitory effect against *E. coli* (16 , 21, 23, 27 mm) respectively while the others bacteria (*P. mirabilis, S. epidermidis*, *K. pneumoniae* and *P. aeruginosa)* inhibitory effect between the bacterial isolates and the concentration of the silver nanoparticles.

Finally, determination of MIC of Ag/AgCl nanoparticles was done by microdilution method. The result showed the MIC for Ag/AgCl NPs for *P. mirabilis, S. aureus, S. epidermidis* and *P. aeruginosa* was 2500 µg/ml while the MIC for *E. coli, K. pneumoniae* was 5000 µg/ml.

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## List of Abbreviation

| Abbreviation | Key |
|---|---|
| **AFM** | Atomic force microscope |
| **Ag/AgCl NPs** | Silver and silver chloride nanoparticles |
| **AHL** | Acyl homoserine lactones |
| **AST** | Antibiotic susceptibility test |
| **Bap** | Biofilm related protein |
| **C.F.C** | Cephalothin, Fucidin, Cetrimide |
| **CDC** | Centers for Disease Control |
| **CFU/ml** | Colony Forming Unit per millimeter |
| **CLSI** | Clinical and Laboratory Standards Institute |
| **CPS** | Capsule polysaccharide |
| **D.D. W** | Double Distal water |
| **D.W** | Distal water |
| **FTIR** | Fourier transform infrared spectroscopy |
| **GRAS** | Generally recognized as safe |
| **HCl** | Hydrochloric acid |

| | |
|---|---|
| **IMViC** | "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. |
| **JCPDS** | Joint Committee on Powder Diffraction Standards |
| **LAB** | Lactic acid bacteria |
| **LPS** | Lipopolysaccharide (Endotoxin) |
| **MDR** | Multiple drug resistant |
| **MIC** | Minimum inhibition concentration |
| **MR** | Methyl red |
| **MRS** | deMan Rogosa Sharpe |
| **nm** | Nanometers |
| **NPs** | Nanoparticles |
| **PDR** | Pandrug-resistant bacteria |
| **Psi** | Pound per square inch |
| **QS** | Quorum sensing system |
| **SCFA** | Short chain unsaturated fats |
| **SEM** | Scanning electron microscope |
| **SPR** | Surface Plasmon Resonance |
| **SWCNTs** | single-walled carbon nanotubes |
| **UTI** | Urinary tract infections |
| **WHO** | World Health Organization |
| **XDR** | Extensively drug-resistant |
| **XRD** | X-ray Diffraction |

# Chapter one
# Introduction

# INTRODUCTION

Infectious diseases is one of the leading causes of morbidity and mortality worldwide. The world health organization (WHO) and centers for disease control (CDC) have expressed serious concern regarding the increase in the development of multidrug resistance bacteria. Therefore, the antibiotic resistance emergency is one of the most problematic issues in global public health. Antibiotic resistance is associated with the lack of new antimicrobials. This has prompted the researchers worldwide to develop novel and effective antimicrobial compounds and to develop novel delivery and targeting strategies (Singer *et al.* 2016).

Bacteria have developed many ways by which they become resistant to antimicrobials. Among those are enzyme inactivation, decreased cell permeability, target protection, target overproduction, altered target site/enzyme, increased efflux due to over-expression of efflux pumps. Other more complex phenotypes, such as biofilm formation and quorum sensing which are related resistance to antibiotics in bacteria (Davies and Davies, 2010).

Different strategies, such as using of nanostructured materials, are being developed to overcome bacteria resistance. Nanostructured materials can be used to convey antimicrobials, to assist in the delivery of novel drugs or eventually, possess antimicrobial activity by themselves. Additionally, using nanoparticles may avoid drug resistance mechanisms in bacteria and associated with their antimicrobial potential, inhibit biofilm formation. Other strategies, including use of plant-based antimicrobials incombination with nanoparticles to overcome toxicity issues, are also being investigated (Laxminarayan *et al.*, 2013). Coupling nanoparticles and natural-based antimicrobials to inhibit the activity of bacterial efflux pumps; formation of biofilms; interference of quorum sensing; and possibly plasmid curing,

are just some of these strategies to combat multidrug resistant bacteria. However, the use of nanoparticles still a challenge to the therapy and much more research is needed in order to overcome microbial resist to antibiotics. Use of generally recognized as safe (GRAS) microorganisms like *Lactobacillus* spp. and other Lactic Acid Bacteria (LAB) strains were used for the synthesis of silver (Ag) nanoparticles (NPs) (Korbekandi *et al*., 2012). The genus *Lactobacillus* are rod-shaped bacteria and they have many strains are commercially available as probiotics with health-promoting properties. It belongs to the group of lactic acid bacteria (LAB). One of the new and prospect areas of LAB applications is nanobiotechnology field. Biological methods of nanoparticle synthesis using bacteria have offered an ecologically friendly and reliable alternative to chemical and physical methods Ag NPs are considered as a valuable alternative for ionic silver and have been widely used as an effective bactericidal agent against pathogenic bacteria, including antibiotic resistant strains (Narayanan and Sakthivel 2010).

**The main objective of this study:**

Biosynthesis of Ag nanoparticles by using local isolates of Lactic acid bacteria and determine the characters, properties and its antibacterial inhibitory effects against multidrug resistance pathogenic bacteria.

These can be established by:

1- Isolation of *Lactobacillus* spp. from the provincial fermented food.

2- Isolation and identification of pathogenic bacteria from different clinical samples in Baqubah Teaching Hospital and Al- Batoul Hospital.

3- Determine the sensitivity of isolated bacteria to classical antibiotics and determine the multidrug resistance antibiotics bacteria.

4- Biosynthesis of Silver nanoparticles (Ag/AgCl NPs) by the CFS (whey) of *Lactobacillus* spp.

5- Study the characteristics of (Ag/AgCl NPs) by using: UV-vis spectroscopy, Atomic force microscope (AFM), Scanning electron microscope (SEM), X-ray Diffraction (XRD) analysis and Fourier transform infrared spectroscopy (FTIR).

6- Study the antibacterial activity of silver nanoparticles on clinical isolate (*in vitro*) and determination of minimum inhibition concentration (MIC) of Ag/AgCl nanoparticles against clinical isolated of pathogenic bacteria.

# Chapter two
# Literature Review

# Literature review

## 2.1 Pathogenesis of bacterial infection

Pathogenesis is a multi-factorial process which depends on the immune status of the host, the nature of the species or strain (virulence factors) and the number of organisms in the initial exposure. A limited number of bacterial species are responsible for the majority of infectious diseases in healthy individuals. The success of vaccination, antibiotics, and effective public health measures, limit the emergence of epidemics. Due to the development of antibiotic resistant organisms, this situation is changing rapidly (Falkow,1997).

Pathogenicity is the capacity to initiate disease. It requires the attributes of transmissibility or communicability from one host or reservoir to a fresh host, survival in the new host, the ability to breach the new host's defenses, and virulence. Virulence in the clinical flied is a manifestation of a complex bacterial–host relationship in which the capacity of the organism to cause disease is considered in relation to the resistance of the host. Types of bacterial pathogens can be classified into two broad groups, primary and opportunistic pathogens. (Casadevall and Pirofski, 2000).

Primary pathogens are capable of causing disease in a healthy individuals with intact immunological defenses. However, these bacteria may more readily cause disease in individuals with impaired defenses. The opportunistic pathogens rarely cause disease in individuals with intact immune system. When such defenses are impaired or compromised, as a result of congenital or acquired disease or by the use of immunosuppressive therapy or surgical techniques, these bacteria will be able to cause disease. Many opportunistic pathogens, e.g. coagulase negative staphylococci and *Escherichia coli*, are part of the normal human flora and are

carried on the skin or mucosal surfaces where they cause no harm and may actually have beneficial effects, by preventing colonization of other potential pathogens. However, introduction of these organisms into anatomical sites in which they are not normally found, or removal of competing bacteria by useing broad-spectrum antibiotics, may allow their localized multiplication and subsequent development of disease (Al-mohanna, 2016).

All humans body are normally colonized with bacteria, the normal flora, living on their external surface including; the skin, gut and lungs. We are constantly also exposed to bacteria (including air, water, soil and food). Due to our defenses mechanism, most of these bacteria are harmless. In compromised patients, whose defenses are weakened, these bacteria often cause opportunistic infectious diseases when entering the bloodstream (after surgery, catheterization or other treatment modalities). When initiated in the hospital, these infectious diseases are referred to as nosocomial. Some common bacteria found in the normal flora include *Staphylococcus aureus*, *S. epidermidis* and *Propionibacterium acnes* (found on the skin). *Bacteroides* and *Enterobacteriaceae* found in the intestine. Due to the development of antibiotic resistant in the organisms and inability to develop new antibiotics in a timely manner to control infection, this attracted the researchers to find natural products work as alternative to antibiotics to treating bacterial infections, especially multiple drugs resistant bacteria ( Davies and Davies, 2010)

In general, pathogenic bacteria are divided into two main parts: Gram-positive and Gram-negative bacteria.

## 2.2 Gram positive bacteria

### *2.2.1 Staphylococcus aureus*

*Staphylococcus aureus* are a gram-positive bacterium that are cocci-shaped and occur in irregular microscopic clusters (looking 'grape-like' henceforth their Greek name Staphylos = grape). Their cell width is 0.5-1.5 mm and have classed with the Staphylococcaceae family. They are facultative anaerobic (with both respiratory and fermentative metabolism), catalase positive and profoundly salt tolerant (most strains can survive in 10% NaCl). *Staphylococcus aureus*, a major human pathogen, causing a large variety of infections worldwide and predominates in surgical wound infections (Kelly, 2012).

*Staphylococcus aureus* on the blood agar plate are convex, large round golden yellow colonies, and frequently produce beta hemolysis. On mannitol salt agar, they produce yellowish halo colonies (Plata *et al*., 2009).

### 2.2.2 *Staphylococcus epidermidis*

*Staphylococcus epidermidis* is gram-positive, coagulase-negative cocci that are a part of our normal flora. Subsequently, it is a true opportunistic, as it requires a major breach in the host's innate defenses. It is one of the main pathogens of nosocomial diseases, especially connected with outside body contaminations. Those most susceptible to infection are intravenous drug users, newborns, elderly, and those using catheters or other artificial appliances. Bacteria produce a glycocalyx "slime" that acts as a barrier to avoid opposition by phagocytosis and killing by some antibiotics. Therefore, they became the major causes of infections colonizing medical implants and cause nosocomial infections worldwide, especially in developing countries ( Rubino *et al*., 2014)

The *Staphylococcus epidermidis* species forms roughly 65-90% of all *staphylococci* isolated from human aerobic flora. Healthy human would possess up to 24 strains of the species. The bacteria is around 0.5 - 1.5 micrometers in distance across. While *S. epidermidis* is a facultative anaerobe, it develops best in vigorous conditions. The bacterial hosts are human and other warm-blooded animals (Nilsson *et al*., 1998).

### 2.2.3 Virulence factors of Staphylococci

*Staphylococcus aureus* and *Staphylococcus epidermidis* produce numerous virulence factors. These elements can be classified into cell-surface-related (adherence) factors and secreted factors (Costa *et al*., 2013).

Catalase: *S. aureus and S. epidermidis* are a catalase enzyme producer, and this protein convert hydrogen peroxide ($H_2O_2$) into water ($H_2O$) and oxygen ($O_2$). Catalase test is critical to recognize staphylococci, which give positive result, from streptococci which are catalase negative bacteria (Jawetz *et al*., 2010).

Coagulase: *Staphylococcus aureus* secrete coagulase, a catalyst which causes plasma clotting in the host's body while *Staphylococcus epidermidis* is negative for coagulase (Quinn *et al.*, 2013).

Hemolysin: *Staphylococcus aureus* is known about its capacity to produce four types (alpha, beta, delta and gamma) which have distinctive antigenic and receptors on the red blood cells. *Staphylococcus epidermidis* produces alpha Hemolysin. Type beta breaks up whole red blood cell in a restricted range, while type alpha causes a less fractional hemolysis (Grumann *et al*., 2014)

Staphylokinase: Staphylokinase or fibrinolysin is produced by all *S. aureus* strains and can disintegrate fibrin clumps which which helps bacterial ease and quick movement in the body, and spread of infection quickly (Hava and Camilli, 2002).

β- lactamase: staphylococci bacteria could produce penicillinase or (beta lactamase), protecting bacteria from ß-lactam antibiotics. The ß-lactam ring of penicillin is cleaved by beta lactamase protein. The wide spread of the catalyst was affirmed by its reality on transmissible plasmids (Murray *et al*., 2007).

Bap: One of the most virulence factor of *S. epidermidis* in biofilm amassing is Bap (biofilm related protein) which is known as a surface bond protein that normally found in *S. epidermidis* strains, while in *Staphylococcus aureus* the causing mastitis isolates are the only strains that harbor the Bap ( Tormo *et al*., 2005 )

Biofilm: A biofilm is a mucous layer of bacterial starting point made out of polypeptide and an external substance such as minerals, nucleic acids, proteins and cell divider content. Biofilm is formed due to bacterial cell exposure to physical conditions, or high density of the cell, etc.. (Burke et al., 2010).

## 2.3 Gram negative bacteria

### 2.3.1 *Proteus mirabilis*

*Proteus mirabilis* is a gram-negative rod-shaped bacterium, it is notable for its urease production and particular capacity to separate into prolonged swarm cells and characteristic bull's-eye pattern of motility on agar plates. *Proteus mirabilis* is belonged to class Gamma proteobacteria, and has for some time been perceived as

an individual of the order Enterobacterial, family Enterobacteriaceae (Adeolu *et al*., 2016).

*Proteus mirabilis*, a portion of the Enterobacteriaceae group of bacilli, is a gram-negative, facultative anaerobe with a capacity to ferment maltose but not ferment lactose. *P. mirabilis* additionally has a swarming motility and the capacity to self-prolong and produce a polysaccharide when it be contact to the solid surfaces; this allows for attachment and easy motility along surfaces (Armbruster and Mobley, 2012).

### 2.3.2 *Escherichia coli*

*Escherichia coli* is a gram negative rod of about (1.1 - 1.5 x 2.0 - 6.0 )µm in size, it grows under aerobic and anaerobic condition (Facultatively anaerobic) due to its two distinctive redox systems (menaquinone and ubiquinone), enable it derive energy from catabolic metabolism under both aerobic and anaerobic condition, with optimal growth. The rate of cell division of the *E. coli* is quick and the quantity of bacterial cells can twofold increase every 20 min. However, the circumstances that are optimum for this population dynamics are not achieved in the bacteria's normal environment. *Escherichia coli* is one of the main causes of nosocomial infections in human (Tobih *et al*., 2006).

### 2.3.3 *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is an individual of the Enterobacteriaceae family a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. *Klebsiella pneumoniae* is able to grow either with or without free oxygen, facultative anaerobe ( Janda, and Abbott, 2006).Virulence of the bacterium is given by group of factors that can prompt disease and antibiotic

resistance. The polysaccharide capsule of the bacterium is the most significant virulent factor, permits the bacteria to avoid opsonophagocytosis and serum executing. Until this point, 77 distinctive capsular types have been contemplated. *Klebsiella* species without a capsule, in general, are less harmful(Shu *et al*.,2009)

### 2.3.4 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a gram – negative bacilli, non-spore forming, non-capsulate and generally motile by one or a couple of polar flagella, estimating about 0.6×2 micrometer. The most widely recognized colonial form is moderately large, low – curved with an unpredictable surface, translucent and an oval shape with the long axis corresponding to the line of inoculum. All strains give a quick oxidase response (inside 30 seconds) which is a valuable invited test for non – pigmented strains .*Pseudomonas aeruginosa* is a strick aerobe; that develops promptly on numerous culture media (Ugur *et al*., 2012)

*Pseudomonas aeruginosa* is catalase and oxidase positive, grow admirably at 37-42°C, its growing at 42 °C recognize it from different *Pseudomonas* species. *Pseudomonas aeruginosa* in a culture can create different colony types (Brooks *et al.,* 2007).

### 2.3.5 Virulence factors of Enterobacteriaceae and *Pseudomonas aeruginosa*

Biofilm development: Biofilm is an assembly of microbial cells on biotic or abiotic surface, encompassed by a polysaccharide material. Biofilm form on a large scale of surfaces, which happens under explicit conditions, helps bacterial existence under numerous harsh conditions and play a significant role in the perseverance of infection (Kwiecinska-Pirg *et al*., 2014).

Capsule: All members of the Enterobacteriaceae are able to elaborate a layer of surface associated polysaccharides called the capsule (Whitfield and Roberts, 1999).

Endotoxin (LPS): Lipopolysaccharide (LPS) are the significant constituent of the external membrane of gram-negative bacteria. They are believed to play a key role in the processes that govern microbial metal binding, surface adhesion and microbe-mediated oxidation – reduction reaction .It protects the bacterial pathogen from host defenses and mediates the entry of the bacteria into eukaryotic cell . It is resistant to phagocytosis and serum, it also acts as a receptor for adsorption of some bacteriophages. It has also a role in the outer membrane permeability barrier (Dunkelberger and Tune, 2010).

Adhesion (Pili and Fimbriae): Bacterial adhesion involves surface interaction between mammalian cell surface (usually carbohydrate), and bacterial surface compound called "ligands" which are protein in nature. The non-specific properties of the bacteria (charge and hydrophobicity) lead to adhesion, and many mechanisms adopted by different bacteria to adhere to mammalian macromolecules or organelles and needs to overcome repulsion forces between the pathogen and target cell. Bacterial adhesion initiate attachment by recognizing host cell receptors located on surfaces of the host cell. Adhesion initiate adherence by overcoming the electrostatic repulsion observed between bacterial cell membranes and surfaces to allow initiate interactions (Ala'Aldeen, 2004).

Hemolysin production: Hemolysin is an extracellular cytotoxic protein cause lysis of red blood cell and lead to harm of tissue, it can destroy the erythrocyte wall and liberate hemoglobin (Liaw *et al.,* 2003).

swarming phenomenon: *Proteus mirabilis* (and different *Proteus* species) has a swarming motility. Swarming motility, is " the movement of highly elongated and flagellated swarm cells across the surface of a solid medium in periodic cycles of movement and consolidation" (Szostek and Rather, 2013).

Urease production: Urease (urea amidohydolase) catalyzes the hydrolysis of urea to deliver ammonia and carbon dioxide, which results in an rise in the pH of urine is produced by *Proteus mirabilis* and *Klebsiella pneumoniae* only. Under these conditions, crystals of calcium and magnesium phosphate precipitate in the urine and produce kidney and bladder stone (Liaw et al., 2003).

Siderophore: are another virulence factor that is required by the organism to cause disease in hosts. Siderophores obtain iron from the host to permit spread of the infection organism (Rønning *et al*., 2019).

## 2.4 Bacteria mechanisms of antibiotic resistance

Resistance is the ability of a bacteria against the antagonize the effect of an antibacterial substances. The development of resistance to antibiotics in bacteria often develop as a result of unnecessary and inappropriate use of antibiotics. Mechanisms are:

**a)** The changes that occur in the receptor that connected to the drug and the region of the connection ‘Connection of the antibiotics’ target areas are different. They can be various enzymes and ribosomes. Resistance associated with alterations in the ribosomal target are the most frequently observed in macrolide antibiotics. Mutations in penicillin-binding proteins (beta-lactamase enzymes) and *Staphylococcus aureus*, can develop resistance to penicillin. Changes in the structure of the target, beta-laktam, quinolones, glycopeptides, macrolides,

tetracycline and rifampicin resistance is an important mechanism in the development (Bassetti *et al*., 2013).

**b)** Enzymatic inactivation of antibiotics: Most of Gram-positive and Gram-negative bacteria synthesize enzymes that degrade antibiotics. Enzymatic inactivation mechanism is one of the most important mechanisms of resistance. In this group, beta-lactamases, aminoglycosides, modifying enzymes (acetylase, fosforiaz adenilaz and enzymes) degrade beta-lactam antibiotics and continually increasing their number of which inactivates enzymes include chloramphenicol and erythromycin (Bassetti et al., 2013).

**c)** Reduction of the inner and outer membrane permeability: This resistance due to changes in the internal and external membrane permeability, decrease in drug uptake into the cell or quickly ejected from the active resistance of the pump systems. As a result of a change in membrane permeability decreased porin mutations in resistant strains can occur in proteins. For example; in *Pseudomonas aeruginosa* strains a specific porin called OprD can cause to mutation carbapenem resistance. Reduction in permeability of the outer membrane may play an important role in resistance to quinolones and aminoglycosides (Nikaido, 1994).

**d)** Flush out of antimicrobial (Active Pump System): Resistance developing through the active pump systems mostly common in tetracycline group of antibiotics. Tetracycline is thrown out with energy-dependent active pumping system and cannot accumulate in the cell. Such resistance is in control of the plasmid and chromosomal. Active pumping systems are effective in resisting quinolones, 14-membered macrolides, streptogramins, chloramphenicol and beta-lactams (Mayer *et al*., 1995).

**e)** Using an alternative metabolic pathway: Unlike some of the changes in the target in bacteria, a new pathway for drug-susceptible eliminate the need to develop objective. In this way resistance seen among the sulfonamide and trimethoprim. Bacteria can gain property of getting ready folate from the environment instead of synthesizing folate (Jawetz *et al*., 1995).

## 2.5 Natural methods to control bacterial infections

The increase of quality of life over the past 50 years is mainly due to the use of antibiotics as antimicrobial chemotherapy. However, antibiotic-resistant bacteria have become a challenging public health problem worldwide (Odonkor and Addo, 2011).The reason may be due to the side effects accompanying antibiotics systemic administration, such as hypersensitivity reactions, kidney problems, liver problems and gastrointestinal upset. Natural health remedies and supplements are undergoing extensive studies to overcome such bacterial resistance to antibiotics and to offer alternative natural antimicrobial agents with least adverse effects on human body . Natural methods of treating bacterial infections began with the use of plant extracts, some types of honey, and then developed using some non-harmful organisms, such as *Lactobacillus* spp. bacteria as probiotic. The genus *Lactobacillus* includes rod-shaped bacteria that are generally recognized as safe (GRAS). They are commercially available as probiotics with health-promoting properties. It belongs to the group of lactic acid bacteria (LAB) (Van *et al*., 2013).

In the last twenty years, there was a great interest in producing nanoparticles as alternatives to antibiotics. One of the new and prospect areas of LAB applications is nanobiotechnology field. Biological methods of nanoparticle synthesis using bacteria have offered an ecologically friendly and reliable alternative to chemical and physical methods (Narayanan and Sakthivel, 2010).

## 2.6 *Lactobacillus* spp.

Lactobacilli are individual from lactic acid bacteria (LAB), an extensively characterized by the arrangement of lactic acid as a type or fundamental final result of starch digestion.It is heterogeneous group with changeful scientific classification, including species with an enormous assortment of phenotypic, biochemical, and physiological properties .This bacteria was isolated by Döderlein in 1892 from the acid vaginal discharge of pregnant lady, this was named Döderlein's bacillus, however it is acknowledged as a strain of *L.acidophilus* (Satokari *et al*., 2005).

Lactobacilli species are phylogenetically diverse, with more than 100 species recorded to date. It is described as Gram-positive, non-spore forming, non-motile, bacilli or coccobacilli in structure , with a G-C content as a rule underneath 50 %.They are robustly fermentative; aerotolerant or facultative anaerobic; aciduric or acidophilic.The aciduric properties of Lactobacilli species are one of their most distinctive merit. lactobacillus is therefore that typically prevail as the last flora in media containing sugar under anaerobic conditions for example vegetables, crushes, milk, cheddar and silage. It is usually alkaline sensitive pH, except for the intestinal types which can resist alkaline pH (Cai *et al*., 2012) .

A few culture media for Lactobacilli have been depicted yet the best and most normal one in the lab scale is deMan Rogosa Sharpe (MRS) medium (Horn *et al*., 2005). Colonies of *Lactobacillus* on agar media are generally little (2-5) mm, with total edges arched, smooth, flashy, and obscure colorless. In uncommon cases, they are yellowish or rosy, a few species from rough colonies, clearly viscid states are just framed by *L.confusus*. Appearance of Clearing zones by exo-enzymes are normally not seen when grow on agar containing scattered protein or lipid, most

strains display slight proteolytic performance because of cell membrane-bound or cell membrane discharged proteases and peptidases .*Lactobacillus* is typically chemoorganotrophic and ferment carbohydrates to produce lactic acid as a major end product (Ezema, 2013).

The growth temperature range of Lactobacilli between 2-35 °C, optimum by and large 30-40 °C, and develop best in marginally acidic media with an underlying pH of 4.5-6.4, development stops with pH of 3.6-4.0 relying upon the species and strain. Also most strain are reasonably aerotolerant, optimum growth is accomplished under microaerophilic or anaerobic condition, expanded $CO_2$ fixation (~5%) may invigorate development .Most *Lactobacillus* develop best at "Mesophilic" temperature with a furthest breaking point around 40 °C, some additionally develop underneath 15 °C and some strain even beneath 5 °C, They supposed "Thermophilic", lactobacilli may have a maximum restriction of 55 °C and don't develop underneath 15 °C. Truly thermophilic lactobacilli becoming over 55 °C are so far unknow (Tannock, 2006).

### 2.6.1 Sources of *Lactobacillus*

Lactobacilli are found in decomposing plant material and fruits, in dairy products, fermented meat and fish, cereals, beets, pickled vegetables, potatoes, sourdough, silages, fermented beverages, juices, sewage and in cavities of humans and animals (Liu *et al*., 2014). In humans, they particularly inhabit the oral cavity, ileum, colon, and are the dominant organisms in the vagina (Djadouni *et al*., 2012).

### 2.6.2 Scientific categorization of *Lactobacillus*

The genus *Lactobacillus* is the biggest genera which remembered for LAB, the genus *Lactobacillus* right now contains 152 known species (Salvetti *et al*., 2012)

and has belong with the phylum *Firmicutes*, class *bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. As per (Salvetti *et al*.,2012) *lactobacillus* is named demonstrated as follows:

Kingdom: Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Lactobacillaceae

Genus: *Lactobacillus*

## 2.7 *Lactobacillus as* Probiotic

Probiotics are live microorganisms which their metabolites have a several antimicrobial impacts on positive and negative gram bacteria. Another word, probiotics are reasonable nonpathogenic microorganisms that colonize the digestive tract, change the intestinal microflora and their metabolic activities emphatically influence the health of the host (Neville and O'Toole, 2010).

For an organism to be a probiotic, it should basically be non-pathogenic (unharmed) endure low pH, and high groupings of conjugated and deconjugated bile salts. They endured by the insusceptible system, and ought not bring about the arrangement of antibodies (Belicova *et al.,* 2013).

Probiotics have numerous advantages for healthy, for example; immunomodulatory impacts of decidedly reactivate, both humoral and cell-mediated immune reactions. Confirmed impacts involve; enhancing production and circulation of serum antibodies, augmenting secretion of cytokines, improving

effectiveness of ingested vaccines and restoring immune function in immunocompromised hosts (Ezendam and van Loveren, 2006). Physiological impacts, involve amelioration of intestinal motility. Antimicrobial effects such as competing for nutrients and adhesion sites with pathogens, lowering intestinal pH, producing short chain of fatty acids (SCFA), and synthesizing extracellular bacteriocins (Gill, 2003).

The members from genus *Lactobacillus* are most ordinarily given safe state, types of this genus including: *L. acidophilus, L. rhamnosus, L. plantarum, L. johnsonii, L. crispatus, L. gallinarum, L. reuteri* are added to nutrition as probiotics (Jeevaratnam *et al*., 2005).

Probiotics are defined as living microorganisms that exert beneficial effects on human health . Such as, treatment of constipation, reduce the risk of colon cancer, alterations in immune function, decrease in pathogenic bacteria, decrease of potential carcinogens, control diarrheal diseases and treatment of lactose intolerance. In addition, Probiotics had beneficial effects as, neutralization of the side effects of antibiotic therapy, lowering of serum cholesterol levels, provide protection against bacterial vaginosis and acquisition of sexually transmitted infections, reduction of endotoxemia from alcoholic liver disease, and treatment of irritable bowel syndrome (Yu – Jing *et al*., 2007).

Beneficial impacts of Lactic acid bacteria (LAB) are two types : direct effects of the live microbial cells, known as the “probiotic influence” or indirect effects throughout fermentation where these microbe’s performance as cell factories for the generation of secondary metabolites with health promoting properties (Hayes *et al*., 2007).

The impacts of probiotics can be arranged in three methods of activity. The first is aligned with the regulation of the host's lines which is in all probability significant for the preservation and treatment of contagious illness and furthermore for the administration of intestinal sore.

The second technique of activity can be called by an immediate effect on different microorganisms which can be commensal and/or pathogenic. In this case, remedy and the treatment of contagion are concerned yet recuperation of the microbial parity in the gut is a significant factor as well . Probiotics have the ability to be rival with pathogens and therefore take into block their grip to the digestive system, while highlighted the probiotics can influence some microbial products (for example, toxins and host products comparative bile salts and nourishment fixings) (Patel *et al*., 2010).

## 2.8 Nanoparticles and Nanobiotics

### 2.8.1 Background

Nanotechnology involves the study of structures at( 1-100) nanometers (nm) which possess novel properties and functions attributable to their small size.These nanoparticles may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine and water treatment."Silver nanoparticles (AgNPs)" are particles which are in the scope of 1-100 nm in size, have extraordinary properties which help in molecular diagnostics, treatments, just as in gadgets that are utilized in a few clinical techniques (van Baarlen *et al*., 2013)

Physical and chemical methods are generally employed for synthesis of silver nanoparticles. The problem with physical and chemical methods is that the

synthesis is expensive and can also have toxic substances adsorbed onto them. To defeat this, the natural procedure gives an attainable other option. The major biological systems engaged with this are bacteria, fungi and plant extracts. The "nano" word is gotten from the Greek "nanos" word concept "dwarf" that point to things of one-billionth around 9-10 nm in the size. The nano-structures have been pulling in more prominent consideration because of the wide potential applications including "nano-medicine" and interesting properties in various fields, that prompted the frugality of straightforward methods for the production and assembly of the nano-sized metal particles. Nanomaterials (NMs) and nanoparticles (NPs) provide solutions to the environmental and technological challenges in several fields and the characteristics are closely dependent upon their overall size, shape, size range composition and distribution (Płaza *et al*., 2014).

## 2.8.2 Properties of nanoparticles

The major parameters of nanoparticles are related to their shape, size and the morphological sub-structure of the substance. Nano-particles are introduced as an aerosol (mostly solid or liquid phase in air), a suspension (mostly solid in liquids), or an emulsion (two liquid phases).Within the sight of chemical agents (surfactants), the surface and interfacial properties might be altered. Indirectly, such laborers can belay against coagulation or aggregation by conserving particle charge ,and by modifying the out layer of the particle (Kazemi *et al.,*2016)

Depending on development history and the lifetime of a nano-particle, the extremely complex structures, perhaps with complex blends of adsorbents must be normal. At the nano-particle fluid interface; polyelectrolytes have been used to adjust surface properties and the associations among particles and their circumference. (Lawrence and Rees, 2012).

⁎ **Nanoparticles can be broadly grouped into two, namely:**

**The organic Nanoparticles (NPs)**: This group incorporates the new allotropic kinds of the carbon that have been found over the most recent two decades. They are utilized in numerous fields of science. Organic NPs implicate, for example, graphene oxide (GO) NPs. Single-walled carbon nanotubes (SWCNTs) and carbon NPs (fullerness) which demonstrated powerful microcidal properties (Vadlapudi and Kaladhar, 2014).

**The inorganic NPs**: these types include; magnetic NPs, honorable metal NPs (gold and silver NPs) and semi-jointer NPs (zinc oxide and titanium oxide). There is a developing attention for inorganic NPs since they are practically flexible with eminent material properties. Metallic NPs are most noteworthy biomedical agents (for example, zinc, gold, carbon, silver, titanium, iron, palladium, fullerenes, and copper) which generally utilized for the synthesis of the NPs. We need to grow ecologically cordial activities through the organic methodologies and the other green synthesis (Vadlapudi and Kaladhar, 2014).

**A few NPs are engaging probes of the natural markers due to: (**Sahayaraj and Rajesh, 2011)**.**

**1-** The little size extending between 1-100 nm.

**2-** The large surface to the volume ratio.

**3-** The biological and chemical features with respect to shape and size strong are affinity to the target especially protein components.

**4-** The structural sturdiness in spite of the atomic granularity.

**5-** The Enhanced or delayed particles aggregation depending on the type of the surface modification and enhanced photoemission.

**6-** Highly heat, electrical conductivity and the improved surface catalytic activity.

**The mechanisms of the activity by which the NPs kill microbes included:** (Xie *et al*., 2011)**.**

1- The production of the reactive oxygen species (ROS) such as $H_2O_2$, $O_2^-$, and $OH^-$.
2- The disorder of bacterial cell wall membrane.
3- The inhibition of the DNA synthesis and intracellular enzymes activity.
4- The interruption of the energy transduction.

Today, by the rise of nanotechnology, numerous NMs and NPs with antibacterial properties have been created to fill the hole antibiotic treatment disappointment (Beyth *et al*., 2015).

## 2.9 Biological Synthesis of Nanoparticles

Green synthesis of nanoparticles is attractive topic of nanoscience and nanotechnology. It includes advancement of perfect, biocompatible, non-lethal and eco-accommodating techniques for nano-particles synthesis when contrasted with traditional strategy like physical and chemical which are frequently poisonous. Biological ways to deal with nanoparticle and nanocrystal synthesis have been stretched out to flawless natural particles. Researchers utilized microorganisms and afterward plant separates for synthesis nano-particles. Nature has concocted different procedures for the synthesis of nano and miniaturized scale length scaled inorganic materials, which have added to the advancement of moderately new and to a great extent unexplored zone of research dependent on the biosynthesis of nano-material's (Kalpana *et al.,* 2017).

Biosynthesis of nanoparticles is a sort of base up approach, where the primary reaction happening is decrease oxidation. The three fundamental strides in the readiness of nanoparticles that ought to be assessed from a green science point of view are, decision of the dissolvable medium utilized for the synthesis, the

decision of a circumferentially considerate reducing agent, and the decision of a nontoxic material for the stabilization of the nano-particles (Wu *et al*., 2015). synthesis utilizing bio-organisms is suitable with the green science standards. The bio-organism is (1) eco-accommodating as may be (2) the reducing operator utilized (3) the capping agent in the reaction (Sankar *et al*., 2015).

Frequently chemical synthesis of nanoparticles lead to the nearness of some dangerous chemical species adsorbed superficially, that may have unfavorable impacts in clinical applications. This isn't an issue with regards to biosynthesized nanoparticles as they are eco-friendly and bio-compatible for pharmaceutical applications. The most bounteous organism in our biosphere is bacteria. This biogenic methodology is incredibly indented with bacteria by giving surrounding conditions, for example, temperature, pH, pressure and so on. The nanoparticles synthesized by natural procedure have higher catalytic reactivity, more prominent special surface territory and furthermore improves the enzyme and metal salt (Suman *et al*., 2013).

Since biological entitles and inorganic materials have steady touch with one another since the time initiation of life on the earth. Biosynthesis is the marvels which occur by methods for biological or enzymatic reaction. Microorganisms produce inorganic materials either intra or extra cell course regularly in nano-scale measurement with impeccable morphology. The extracellular creation of nano-particles has increasingly business as contrast with intracellular procedure. As indicated by Beveridge,1997 the mechanisms which are considered for the biosynthesis of nano-particles included efflux system, modification of solubility and poisonous quality by means of reduction or oxidation, bio-absorption, bio-accumulation, extracellular appearance or precipitation of metals, and absence of special metal transportation system (Rai *et al*., 2011).

## 2.10 Applications of Silver nanoparticles

Among numerous metals, silver has been utilized previously as metallic silver, silver nitrate, and silver sulfadiazine. In any case, because of the appearance of numerous anti-infection agents, the utilization of these silver mixes has been remarkably little. The silver has been utilized as AgNPs, demonstrating the critical antimicrobial action against the multi-drug resistant microorganisms due from their decreased size. Recently, nano-encapsulated therapeutic agents have been utilized to selectively target anti-tumour agents, thus, resulting in higher drug concentrations at the tumors site (Al-Sheddi *et al*., 2018)

Silver nano-particles (AgNPs) are sort of material with a few applications, for example, sensors, anticancer operators, catalysts and antimicrobial agent. AgNPs have displayed activity against bacteria, fungi and viruses Silver nano-particles are significant on account of their unrivaled properties, (for example, optical, electrical, and magnetic properties) which can be consolidated into antimicrobial applications, biosensor materials, cryogenic superconducting materials, composite fibers, corrective items, and electronic segments (Kashyap *et al*., 2013).

### 2.10.1 Silver nanoparticles as antimicrobials

Silver component have been utilized to treat wounds, burns and diseases. Different salts of silver and their subsidiaries are utilized as antimicrobial agents. Numerous examinations have detailed that nano measured silver particles have antimicrobial properties (Xiu *et al.,* 2012). Nano-particles of silver have been studied as a medium for anti-biotics delivery and to production composites for use as sterilizing filters and covering materials (Tran *et al*.,2013).

A few systems have been proposed to clarify the inhibitory impact of silver nano-particles on bacteria. It is expected that the high liking of silver towards

sulfur and phosphorus, is the key component of the antimicrobial impact, because of the wealth of sulfur containing proteins on the cell membrane of bacteria (Ramalingam *et al.,* 2016). Silver nano-particles can interact with sulfur containing amino acids inside or outside the cell membrane which thus influences bacterial cell viability. It was additionally proposed that silver particles (especially Ag+) discharged from silver nano-particles (AgNPs) can connect with phosphorus moieties in DNA bringing about inactivation of DNA replication or can react with sulfur containing proteins and causing suppression of enzymes activities (Mitzel ,2017*).*

The silver nano-particles of under 10 nm widths appended to the bacterial cell wall causes apertures in the cell wall which prompts the cell decease, this examination recommends that the method of silver nano-particles activity, is that the AgNPs get joined to the sulfur containing proteins on the bacterial cell wall prompting expanded penetrability of the layer, at long last; causing cell passing. The potion subordinate impact of silver nano-particles in the size scope of 10-15 nm on the negative and positive gram bacteria has been considered (Agnihotri *et al*., 2014).

At smaller scale molar degrees of Ag+ particles have been denounced to uncouple respiratory electron transport from oxidative phosphorylation, restrain respiratory chain proteins and interpose with the layer penetrability to protons and phosphate (Sukhorukova *et al*., 2017). Moreover higher condensation of Ag+ particles have been appeared to associate with cytoplasmic ingredients and nucleic acids .The impact of silver nano-particles on the cell morphology of *E. coli* and *S.aureus* has been Studied utilizing TEM, SEM and X-ray microanalyses .It was uncovered that treatment with the silver particles bring about similar

morphological changes in both the positive and negative gram bacteria. ( De Giglio *et al*., 2013).

The antibacterial activity was molecule size dependent, the silver nano-particles likewise display shape subordinate interaction with the bacterial cells. The shortened triangular silver nano plates showed the most powerful biocidal activity against *E. coli* when than the circular and rod form nanoparticles (Wilke *et al.,* 2018)

Small size particles with bigger surface to volume proportions have more noteworthy antibacterial efficiency. Nanoparticles with size under (25) nm against highly multi-resistant bacteria, for example, methicillin resistant *Staphylococcus aureus*, methicillin-resistant coagulase-negative staphylococci (as, *Staphylococcus epidermidis*), vancomycin resistant *Enterococcus faecium*, and ESBL positive *Klebsiella pneumoniae*. This is a significant outcome especially when antibiotics resistance among bacterial species is expanding at a disturbing rate and not many choices are accessible to address the issue. Silver nano-particles have been assessed for their antimicrobial exercises against a wide scope of pathogenic life forms (Rudramurthy *et al.,* 2016).

## 2.11 Toxicity of nanoparticles

The metal nanoparticles have broad manufacturing and clinical applications because of their novel physicochemical and biological properties. However, there is a shortage of information about the impact of the extended period exposures to nano-particle on human health and ambience. The ramifications of nano-particles on health and ambience should be evaluated totally before their enormous scope creation and application in different fields (Bouwmeester *et a*l., 2009).

Cell culture based techniques are utilized as a prescreening device to comprehend the biological impacts of nano-particles. Anyway alongside in vitro measures it is important to affirm in the *vivo* the biological efficacy of nano-particles in animals models, to contemplate the appropriateness of their applications. It is clear that metal based nano-particles due to their physiochemical and biological properties are promising as anti-microbial and therapeutic operators. They can be utilized to address various difficulties in the field of nano-medication. However; it must be recollected that they can likewise cause unfavorable natural impacts, at the cellular and subcellular levels. Along these lines, after the cytotoxicity and clinical investigations, the nano-particles can discover massive application as anti-microbial in the medical and industrial fields. (Schrand *et al*., 2010).

# Chapter three
# Materials and Methods

# Materials and Methods

## 3.1 Materials

### 3.1.1 Laboratory Instrument's and equipment's

The Instruments and equipment used in this study are listed in table (3-1).

Table (3-1): Instruments and equipment used in the study.

| **Instruments and equipment** | **Company (country)** |
|---|---|
| Atomic Force Microscopy (AFM) | AA-3000, Shimadzu(Japan) |
| Autoclave | Hva-85 Hirayama (Japan) |
| Centrifuge | Gallenkamp (England) |
| Compound light microscope | Olympus(Japan) |
| Electric Sensitive balance | Mattler (Switzerland) |
| Fourier Transform Infra-red Spectroscopy (FTIR) | Shimadzu(Japan) |
| Incubator | Binger (Germany) |
| Micropipette | Oxford (U.K) |
| Oven | Memmert(Germany) |
| Penner | Slibrand (China) |
| pH meter | Jenway(USA) |
| Scanning Electron Microscope (SEM) | Inspects50 (Holland) |
| Spectrophotometer | Gallenkamp (England) |
| Sterilized cotton swabs | Sterellin Ltd. (UK) |

| | |
|---|---|
| UV-Vis Spectroscopy | UV-1800, Shimadzu(Japan) |
| VITEK 2 Compact system | BioMerieux(USA) |
| Vortex | Memmert(Germany) |
| Water bath | Memmert |
| X-ray diffractometer (XRD) | Bruker D8, Karlsruhe (Germany) |

### 3.1.2 Chemicals and Biological Materials

The Chemicals and biological materials used in this study are listed in table (3-2).

Table (3-2): Chemicals and biological materials used in the study

| **Chemicals and biological materials** | **Company(country)** |
|---|---|
| Deionized water | Iraq |
| Glycerol | BDH |
| Hydrochloric acid (HCl) 40% | BDH (England) |
| Silver nitrate 99% ($AgNO_3$ M.W 169.88) | Sigma(USA) |

### 3.1.3 The dyes

Dyes used in this study are listed in table (3-3).

Table (3-3) Dyes used in this study

| Dyes | Company(country) |
|---|---|
| Crystal violates, iodine, absolute alcohol, and safranine (Gram stain set). | BDH (England) |
| Methyl red | BDH |
| N, N,N,N-tetramethyl-P-phenylene-diamine dihydrochloride(TMPD , Oxidase reagent | BDH |

### 3.1.4 The biochemical solutions

Ready biochemical solutions used in this study are listed in table (3-4)

Table (3-4) Ready biochemical solutions used in this study

| Biochemical solutions | Company(country) |
|---|---|
| Standard MacFarland's solution (matching a turbidity of $0.5\times10^{8}$ CFU/ml ) | Bio Mérieux (France) |
| Kovac's reagent | Fluka chemika(Switzerland) |
| Catalase reagent | BDH (England) |
| Vogas-Proskauer reagent | BDH |

### 3.1.5 Culture Media

The Culture media used in this study are listed in table (3-5).

Table (3-5): Culture media used in this study.

| Culture media | Company(country) |
|---|---|
| Blood agar | Himedia (India) |
| Brain heart infusion broth | Himedia |
| Eosin methylene blue (EMB) | Himedia |
| Lactobacillus MRS agar | Himedia |
| Lactobacillus MRS broth | Himedia |
| MacConkey agar | Himedia |
| Mannitol Salt Agar | Himedia |
| MR-VP broth | Himedia |
| Muller-Hinton agar | Himedia |
| Nutrient agar | Himedia |
| Nutrient broth | Himedia |
| Peptone water | Himedia |
| Pseudomonasagar + C.F.C supplement(Cetrimide,Fucidin, Cephaloridine ) | Himedia |
| Simmon's citrate agar | Himedia |
| Urea agar base | Himedia |

### 3.1.6 The Antibiotics

The Antibiotic used in this study are listed in table (3-6).

Table (3-6): The antibiotic disk used in this study.

| Antibiotics | Abb. | Conc. (µg/ disk) | Class of antibiotics | Company (Country) |
|---|---|---|---|---|
| Ciprofloxacin | CIP | 5 | Fluoroquinolones | Mast group LTD Merseyside. (U.K) |
| Ofloxacin | OFX | 5 | | |
| Levofloxacin | LEV | 5 | | |
| Gentamicin | GM | 10 | Aminoglycoside | Mast group LTD Merseyside. |
| Amikacin | AK | 30 | | |
| Streptomycin | S | 10 | | |
| Tobramycin | TN | 10 | | |
| Piperacillin | PRL | 100 | Penicillin | Bioanalyse (Turkey) |
| Doxycycline | DXT | 30 | Tetracyclines | Mast group LTD Merseyside. (U.K) |
| Tetracycline | T | 30 | | |
| Trimethoprim | TM | 5 | Folate pathway antiagonists | Mast group LTD Merseyside. |
| Trimethoprim-sulfamethoxazole | STX | 1.25/ 23.75 | | |

| | | | | |
|---|---|---|---|---|
| Cefotaxime | CTX | 30 | Cephems including cephalosporins 1,2,3,4. | Mast group LTD Merseyside. (U.K) |
| Cefuroxime | CXM (CFX) | 30 | | |
| Cefoxitin | FOX | 30 | | |
| Ceftazidime | CAZ | 30 | | |
| Cefepime | CPM | 30 | | |
| Vancomycin | VA | 30 | Glycopeptides | Mast group LTD Merseyside. |
| Nitrofurantoin | NI | 300 | Nitrofurans | |
| Clindamycin | CD | 2 | Lincosamides | |
| Imipenem | IMI | 10 | Carbapeneams | Mast group LTD Merseyside. |
| Meropenem | MEM | 10 | | Bioanalyse (Turkey) |
| Azithromycin | ATH , AZM | 15 | Macrolides | Mast group LTD Merseyside. (U.K) |
| Clarithromycin | CLA | 15 | | |
| Chloramphenicol | C | 30 | Phenicol | |
| Cefpodoxime | CPO | 10 | Cephems(oral) | Mast group LTD Merseyside. |
| Ampicillin-sulbactam | AMS | 10/10 | β-Lactam combination agents | Mast group LTD Merseyside. |
| Ticarcillin-clavulanate | TCC | 75/10 | | |

| | | | | |
|---|---|---|---|---|
| Aztreonam | ATM | 30 | Monocyclin | Mast group LTD Merseyside. (U.K) |

### 3.1.7 Human blood

Human blood used in this study was obtained from the Baqubah Hospital /blood bank.

## 3.2 Methods

### 3.2.1 Sterilization methods

#### 3.2.1.1 Sterilizing different types of media

The moist heat sterilization method was by using autoclave for 15 min. at 121°C and under pressure 15bar/in$^2$ (Atlas *et al*., 1995).

#### 3.2.1.2 Sterilizing different types of Tools

The dry heat sterilization method was by using oven for 2 h at 180 °C to Glassware sterilizing, while needles and loops were sterilized by penner (Atlas *et al*., 1995).

### 3.2.2 Preparations of culture media

#### 3.2.2.1 Ready-made media

Ready-made media include: MacConkey agar, Brain heart infusion broth, Muller-Hinton agar, Simmon's citrate agar, MR-VP broth, Urea agar base, EMB, Nutrient agar, Nutrient broth, Peptone water, Lactobacillus MRS broth, Lactobacillus MRS agar and Mannitol Salt Agar were prepared according to the instruction of a manufacturing companies. The culture media were sterilized by autoclaving. They

were incubated for 24 h at 37°C to ensure their sterility after being prepared, than kept at 4°C till they were used.

### 3.2.2.2 Laboratory prepared media

#### 3.2.2.2.1 Blood agar medium

Blood agar medium was prepared by dissolving 40gm of blood agar base powder in 1000ml of distalled water, then it was heated to dissolved and sterilized by autoclaving at 121°C for 15 minutes under 15 psi. After cooling to 50°C, fresh human blood 5% was added to the media and poured into sterilized petri dishes (Forbes *et al*., 2002)

#### 3.2.2.2.2 Pseudomonas agar

It was set up by dissolving 24.2 gm of pseudomonas agar base in 100 ml at that point total to 500 ml with pH was 7 at that point warmed until the constituent is totally broken up. The media was sterilized by autoclaving at 15 Psi pressure, 121C° for 15 min. The culture media were cooled to 50 C° and aseptically add sterile Cephalothin, Fucidin, Cetrimide (C.F.C) vial supplement (as needed). Then, the media were blend well and add into sterile Petri plates, which were utilized for isolation of *Pseudomonas* (Alfred, 2005).

#### 3.2.2.2.3 Urea agar

It was set up by Suspend 24 gm of the urea agar base medium in 950 ml distilled water Sterilize by autoclaving paragraph ( 3.2.1.1), after cooling at about 45ºC, add 50 ml of urea 40% and mix well. Then dispense 5 ml in test tube and set at slant position.

### 3.2.3 Collection of clinical specimens

In this study, a total of 200 swabs and samples were collected from burns, wounds and urine specimens from Baqubah Teaching Hospital and Al-Batoul Hospital during a period of November 2019 to January 2020.The specimens were taken by a sterile swab with culture media(burns and wounds ) and urine sample from patients suffering from acute and chronic infections.

### 3.2.4 Identification and laboratory diagnosis of bacteria isolates

### 3.2.4.1 Morphological examination

Morphological examination based on the morphological characteristic (as primary diagnostic tests) of isolates grown on MacConkey agar and Blood agar (incubated at 37°C for 24h) were studied including colony color, shape, edges, specific material production, blood hemolysis and texture (Holt *et al*., 1994).

### 3.2.4.2 Microscopical examination

Microscopic examination is done by taking one bacterial colony and transported to the microscopic slide, fixed by quick pass over a penner flame then stained using Gram stain technique. Cell arrangement and shape were observed (Holt *et al*., 1994).

### 3.2.4.3 Identification by subculture on selective and Differential media

Some of the isolated bacteria were subcultured on selective and differential media as follow: *E. coli* was subculture on the Eosin methylene blue (EMB) agar that prepared according to the manufacturing company instructions and sterilized by autoclaving paragraph ( 3.2.1.1).

* *P. aeruginosa* was subculture on Pseudomonas agar that was discussed in paragraph (3-2-2-2-2).

**S. aureus* and *S. epidermidis* were subcultured on Mannitol Salt agar medium which was used as a selective media for Staphylococci and Micrococci as well as differentiation of *S. aureus* from *S. epidermidis* (Macfaddin, 2000). The culture medium was prepared according to the manufacturing company instructions and sterilized by autoclaving paragraph ( 3.2.1.1).

### 3.2.4.4 Identification by Biochemical tests

### 3.2.4.4.1 Oxidase test

It is used to identify bacteria that produce cytochrome C oxidase; an enzyme of the bacterial electron transport chain. The cytochrome-C oxidase oxidizes reagent (tetramethyl-p-phenylenediamine) to (indophenols) giving purple color as end product. The tested colony was transferred by sterile wooden stick to a filter paper, then few drops of oxidase reagent were added. A purple color should develop during 10-30 seconds in a positive reaction. The color of the colony remains the same in case of negative result (Prescott, 2002).

### 3.2.4.4.2 Catalase test

Catalase is an enzyme produced by microorganisms that live in aerobic environments to neutralize toxic forms of oxygen metabolites $H_2O_2$ . The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide. Anaerobes bacteria lack this enzyme. The tested colony was transferred with a sterile wood stick onto a clean glass microscope slide, then few drops of 3% hydrogen peroxide $H_2O_2$ were added; the release of oxygen bubbles indicates the presence of catalase (Collee *et al*.,1996).

### 3.2.4.4.3 IMiVC test

All the biochemical IMiVC tests were carried out according to (Collee *et al*., 1996).

#### 3.2.4.4.3.1 Indole test

Indole test was used to determine the ability of an organism to split amino acid tryptophan by tryptophanase and form indole. Peptone water was inoculated with overnight tested bacterial culture and incubated at 37 °C for 24-48 hr. After that, 0.5 ml of Kovac reagent was added directly to the culture vial. The immediate formation of a red ring at the top of the broth indicates positive result.

#### 3.2.4.4.3.2 Methyl red test

Methyl red (MR) test is determining microbe that performs mixed acids fermentation when metabolite glucose. In mixed acid fermentation, three acids (lactic, acetic and succinic) are formed in significant amounts. These large quantity of acid results significant decrease in the pH of the medium below 4.4. This is visualized by using pH indicator, methyl red is yellow above pH (5.1) and red at pH (4.4).MR-VP medium was inoculated with the tested bacterial culture and incubated at 37°C for 72 hr. Few drops(5-6) of methyl red solution were added to the broth culture. An immediate red color reaction indicates positive result but yellow reaction means a negative result.

#### 3.2.4.4.3.3 Vogas-Proskauer test

This test was used to determine the ability of an organism to produce acetoin as the main end product of glucose metabolism and form smaller amounts of mixed acids. In the presence of oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol act as a catalyst to bring out a red

complex. MR-VP broth medium was inoculated with the tested bacterial culture, incubated at 37℃ for 24-48 hr.; then 3 ml of solution 1 and 1 ml of solution 2 previously prepared were added; red color appearing after 5 minutes indicates a positive result.

### 3.2.4.4.3.4 Citrate Utilization test

This test was used to identify the ability of bacteria to utilize sodium citrate as the only carbon source. The inorganic ammonium dihydrogen phosphate ($NH_4H_2PO_4$) is the only fixed nitrogen source. Simmon's citrate agar slant was inoculated with tested bacterial culture by streaking with sterile loop and incubated at 37℃ for 24-48 hr. The positive result was indicated by changing the color of the medium from green to blue.

### 3.2.4.4.4 Kligler's iron test

Kligler's iron test (KIA) was used to detection the carbohydrate fermentation. Reactions of KIA helps to identify a particular bacterial isolate of the Enterobacteriaceae family. If an organism can't ferment glucose, then alkaline slant-alkaline-butt (no change) reaction was appeared. This reaction alone,is sufficient to exclude an isolate from the Enterobacteriaceae family. Kligler's iron slant was inoculated with tested bacterial culture by streaking on the surface and stabbing in butt of the medium, then incubated at 37℃ for 24-48 hr. The positive result was noticed by changing the color of the culture medium indicator and production of $H_2S$.

**⁎For slant and butt:**

Positive test: yellow (acid)

Negative test: red (alkaline)

* For butt:

Positive test: yellow

Negative test: red to brown

Red slant/yellow butt: dextrose positive, lactose negative

Yellow slant/yellow butt: dextrose positive, lactose positive

Red slant/red butt: dextrose negative, lactose negative

* $H_2S$ Production:

Positive test: Black precipitate or color throughout the culture medium or at the junction between slant and butt

Negative test: No black color development (Collee *et al.*, 1996).

### 3.2.4.4.5 Urease production test

The inoculated tubes that contain urea agar were prepared according to company's instructions. The culture media were stabbing and streaking on slant and incubated in 37°C for 24 hours. The conversion of medium to pink color refers to a positive result (Marin *et al*., 2002).

### 3.2.4.4.6 Confirm the identification of bacterial isolate by VITEK-2 system:

A specific number of bacterium isolate was selected to confirm identification, the Vitek-2 system. The Vitek-2 system consists of an instrument and a computer, and the instrument consists of 5 essential components:

1- Keypad.

2- Fill Door: where the sample was transmitted form the Khan tube into the kit by transmission pipeline in the kit. This process continued for 70 seconds.

3- Load Door: The transmission pipeline located on the kit was cut and transferred into incubator, this process continued for 3-5 minutes.

4- User access door: where the incubation and measures changes occurred to the kit due to growth of bacteria in order to get a result.

5- Waste Door: used to collect kits after finishing the analysis and obtaining the result.

The Vitek-2 system was used to confirm the diagnosis of isolates and do sensitivity test for antibiotics that were used to diagnose most types. Each sample needs two kits, one of them was used in diagnosis and the other used for sensitivity test. The diagnosis kit contained 64 wells. Inside each well, there was a dried substance and colored indicator. The Vitek-2 compact system recorded the color change as a result of bacterial growth, and the diagnosis kit of sensitivity test contained (18-20) antibiotics distributed on 64 wells, so that each antibiotic has more than a single concentration and the Vitek-2 system recorded the change in turbidity after growth of bacteria. These tests were performed as follows:

1-Prepartion of inoculum:

The isolated bacteria were inoculated on nutrient agar by streaking method, incubated at 37 °C for 18-24 hours. Then one Khan tube was taken for sensitivity test and 3 ml of normal saline was added by a micropipette. Then, a loopful was taken and transferred to Khan tube to obtain suspension for diagnosis tube, and transferred to fixed value from the diagnosis tube to sensitivity test tube with a micropipette from suspension (280 microliter).

2-After sample preparation, it was inserted into fill door to transfer the sample of Khan tube into a kit (cassette), then the sample was transferred manually to the

load door for incubation, and then the results of sensitivity to antibiotics were measured between (6-12) hours (David ,2011).

### 3.2.5 Isolates preservation

#### 3.2.5.1 Short term maintenance

Each bacterial isolate was preserved after the insurances of their purification by streaking a slant of nutrient agar with each bacterial isolate and was incubated at 37°C for 24 h. Slants were kept at 4 °C for few weeks (Harly and Prescott, 2002).

#### 3.2.5.2 Long term storage

The bacterial isolates were stored for a long time in a medium containing 20% glycerol at -20 °C (freezing). The medium consists 8.5 ml of brain heart infusion broth in the Fogged tube, then sterilized by autoclave. After cooling, the media was inoculated with pure bacterial isolated colony and incubation for 24h. at 37°C, and then a 1.5ml of glycerol added to the tube (final volume of 10 ml) and the tubes were stored at -20 °C (Ausubel *et al*., 1987).

### 3.2.6 Antimicrobial susceptibility test

All the bacterial isolates were tested for antimicrobial susceptibility according to the CLSI (2019) criteria using agar diffusion method as follows:

⁎Few bacterial colonies (2-4) from overnight culture were transferred to 2 ml of normal saline to prepare the bacterial suspension which was adjusted to McFarland turbidity ($1.5\times10^{8}$) CFU/ml.

⁎ Bacterial suspension was inoculated in Muller-Hinton agar plates by using a sterile cotton swab. The plates were left to dry.

∗ Different antimicrobial discs table (3-7) were used with a maximum six discs placed on the surface of Muller-Hinton agar media. The plates were wrapped with parafilm to prevent any possible contamination during incubation and then incubated for 24 h at 37°C.

∗The diameter of the inhibition zone of each antibiotic disc was measured and the results were interpreted by referring to CLSI (2019) recommendation.

Table (3-7) the antibiotics that used for each bacterium isolate

| No | Bacteria isolates | Antibiotics used |
|---|---|---|
| 1 | *E. coli* | Imipenem,Tetracycline, Doxycycline, Aztreonam,Levofloxacin,Cefpodoxime ,Trimethoprim-sulfamethoxazole ,Azithromycin,Ampicillin-sulbactam Ticarcillin-clavulanate,Cefoxitin, Cefuroxime |
| 2 | *S. aureus & S. epidermidis* | Ciprofloxacin, Ofloxacin, Gentamicin, Vancomycin,Clarithromycin, Tetracycline, Doxycycline, Clindamycin Streptomycin, Chloramphenicol, Azithromycin, Levofloxacin |
| 3 | *K. Pneumoniae & P. mirabilis* | Ciprofloxacin, Ofloxacin, Gentamicin, Trimethoprim, Cefotaxime, Cefuroxime, Amikacin, Streptomycin Nitrofurantoin, Piperacillin, Imipenem, Chloramphenicol |

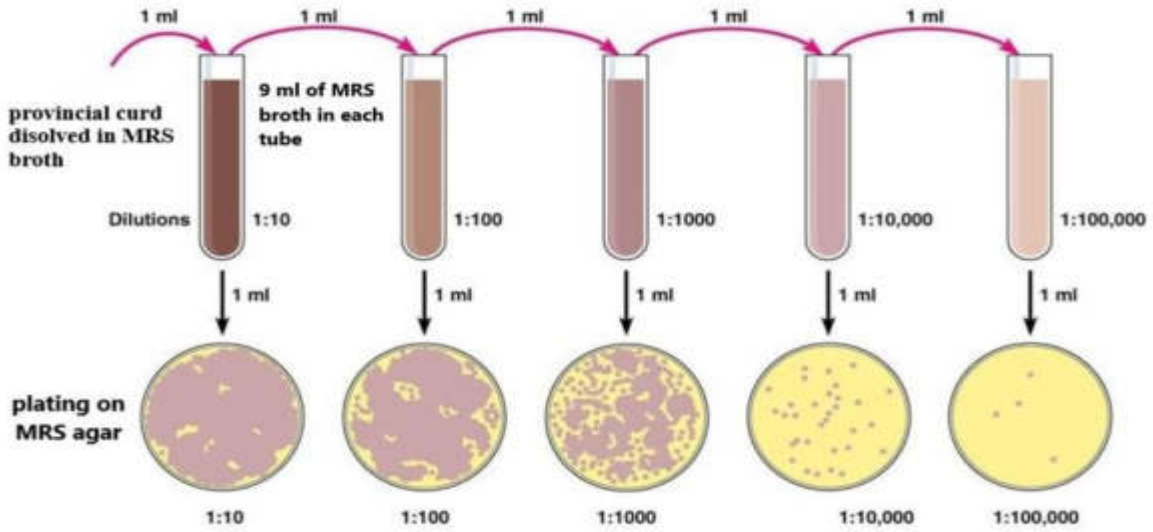
| | | |
|---|---|---|
| **4** | ***P. aeruginosa*** | Ciprofloxacin, Ofloxacin, Cefepime, Aztreonam, Gentamicin, Amikacin, Piperacillin, Imipenem, Levofloxacin, Meropenem, Ceftazidime, Tobramycin |

* The underlined antibiotics refer to the only used antibiotic that is differ between bacteria in susceptibility test.

### 3.2.7 Isolation of *Lactobacillus* spp.

The samples of local fermented and curd food (dairy products) were collected from various local markets in Baqubah city.

1. *Lactobacillus* isolates, which used in the study, were isolated by serial dilution technique using MRS Broth and Agar (Ranganath *et al*., 2012) as shown in figure (3-1), where the culturing was done by pouring the dishes and then incubated under microaerophilic conditions with $Co_2$ (5%) by use candle jar for 48h.
2. The bacterium was identified based on cultural and biochemical characteristics.
3. Initially, all of the isolates were examined for Gram staining and catalase production. Then, the cell morphology and colony characteristics on deMan Rogosa and Sharpe agar (MRS agar) were tested and the isolates were separated into different phenotypic groups.
4. Only Gram-positive, catalase-negative and rod shaped isolates were selected for further studies.
5. The isolates were maintained in slants of MRS agar.




Figure(3-1) Serial dilution technique using MRS Broth and Agar to isolate *Lactobacillus* spp.

### 3.2.8 Screening of *Lactobacillus* spp. for silver nanoparticle biosynthesis

In a typical procedure of nanoparticles biosynthesis:

1. *Lactobacillus* spp. isolates were inoculated into sterile 250 ml of home delivered milk (skim milk) into 500 ml Erlenmeyer flask, for curdling at 37°C for 24 hours.
2. The whey was collected by coarse filtration (Whatman 40). The filtrate was pale yellow in appearance, and the pH was typically (4.4).
3. The presence of lactic acid bacteria in the supernatant was confirmed by observation taken from optical microscope.
4. Five mL of each filtrate whey solution taken in a test tube, 1 mg of $AgNo_3$ was added and kept in the laboratory under ambient conditions (Nair and Pradeep, 2002).

5. The solution became brown in about 12 h. A brown mass gets deposited at the bottom of the test tube after 24 h.
6. The control was run along with experimental tubes.

The underneath scheme shows one of the reduction strategies for nanoparticles blend. (Figure 3-2).

**Stage 1:** Isolated the particular microbial isolates and culture them into special supplement media (MRS)

**Stage 2:** Added of the identical metal salt into culture medium of which the nano-particles were required

**Stage 3:** Preserved the culture media at special pH and temperature and permitted the chelating redox reaction to happen

**Stage 4:**Tint change of media mirror the physical sign of nanoparticle production, this can be additionally checked utilizing UV visible range.

**Stage 5:** Gathering nano-particles: portrayed utilizing SEM, UV, XRD,FTIR and AFM

**Stage 6:** Purification of nano-particles which can be utilized for applications dependent on their basic size limit.

Figure (3-2): Generalized flow chart for biosynthesis of nanoparticles using microorganisms

### 3.2.9 Harvesting the possible biosynthesized of AgNPs

After the precipitate was formed, each tube containing the precipitate was centrifuged to ensure sedimentation of all the nanoparticles. Centrifugation was done at 8000rpm for 5 minutes. After that, the leachate was eliminated carefully by using auto-micropipette. The remainder of solution with the precipitate in the tube was violently mixed and dispersed in a glass flask. In order to obtain a pure powder. First, the product was dried at 50 ° C for 24 hours, and then was calcination at 150° C for 3 hours. The calcination process was carried out according to Goudarzi *et al*.(2016) who found that process with a temperature range of (300- 600) °C causing an increase in the size of the nanoparticles. Therefore, the powder was exposed to a low temperature to avoid affecting the size of the nanoparticles. In order to get rid of any unwanted material residues, especially organic materials, the nanoparticle production process was carried out in a biological production method.

### 3.2.10 Characterization of Silver Nanoparticles

The characterization of Ag NPs was carried out by using X-ray diffractometer (XRD) pattern analysis (The Ministry of Science and Technology/ Baghdad). The molecular analysis of the samples was performed by Fourier Transform Infra-Red Spectroscopy (FT-IR) and UV-Visible Spectrophotometer in the Physics department\Collage of Sciences\Diyala university. The morphology analysis and particle size of samples were carried out by Emission Scanning Electron Microscope (SEM) Physics department\Collage of Sciences \Basrah university, The size, topography and granularity volume distribution of biosynthesized nanoparticles are characterized by the use Atomic Force Microscopy (AA-3000, Shimadzu Japan), (characterized by Dr.Abdul Kareem Al-Samaraii Lab. Baghdad/Iraq.

### 3.2.11 Determination the antibacterial activity of Silver nanoparticles in *vitro*

Determination of Silver nanoparticles antibacterial activity was done by agar wells diffusion assay according to Obedat *et al*. (2012) with some modification. Antibacterial activity was determined against multiple drugs resist (MDR) bacteria; three species of Enterobacteriaceae bacteria *(P. mirabilis , E. coli ,K. pneumoniae)* which were isolated from urine and burn infections and three species (*S. aureus S. epidermidis* , *P. aeruginosa )* which were isolated from wound and burn infections**.**

**1-**The first a stock of AgNPs 100% was prepared by dissolved 280 mg of NPs powder in 2.8 ml of deionized water (the dissolve process was done by using vortex and heating the solution in water bath (40 °C to make sure that all powder was dissolved completely).

**2-**Four different concentrations were prepared from the stock; 12,500 ; 25,000; 50,000 ; 100,000 µg/ml.

**3-** Each bacterium was cultured on Muller Hinton agar after comparison with McFarland tube ($1.5x10^8$) CFU/ml by streaking method and the five wells 5mm were made in the plate by sterilized cork borer.

4- The four different concentrations of silver nanoparticles 100 µl were added to wells, the fifth well was taken as control by add 100 µl of deionized water. As shown in figure (3-3).

5- Three repeats made for each bacterium and then incubated at 37 °C for 24 hours.

6-The efficacy of each concentration of nanoparticles was determined by measuring the inhibiting diameter of each concentration by a standard ruler in millimeters.

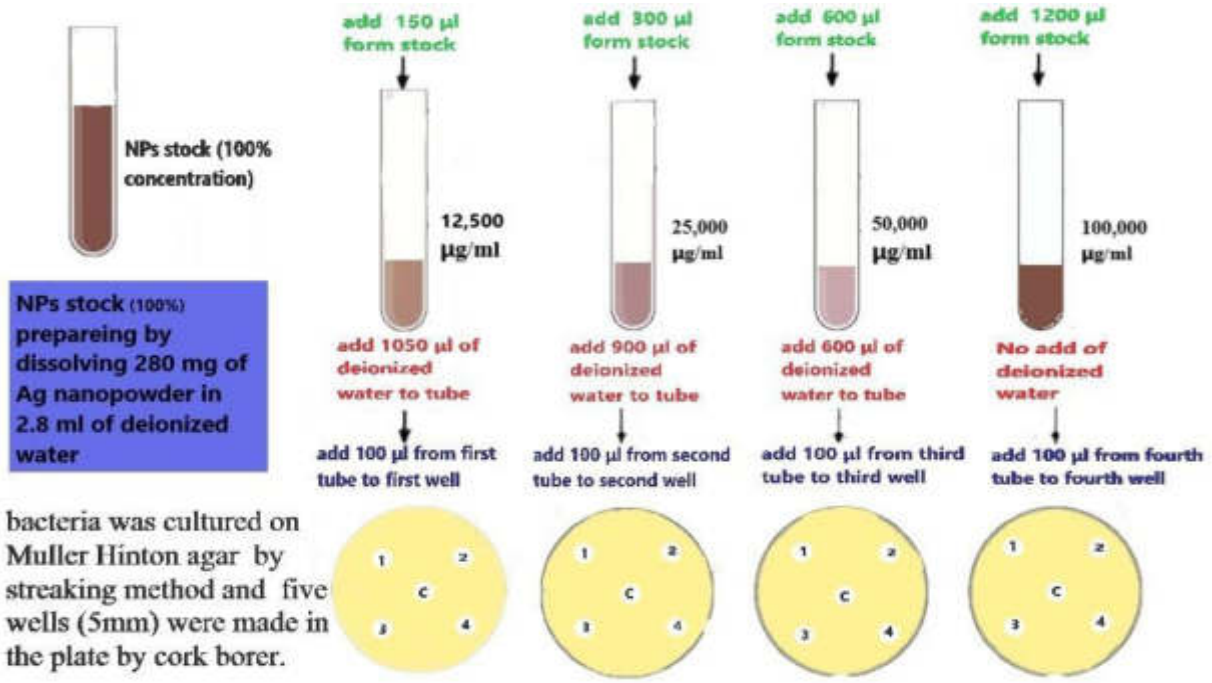



Figure (3-3) determination of Silver nanoparticles activity by agar wells diffusion assay

## 3.2.12 Determination the Minimum Inhibitory Concentration (MIC) of Silver nanoparticles

Since MIC was the most minimal concentration of an antimicrobial agent that repress the growth of microorganisms after overnight culture. MIC was essential for the determination or to confirm the resistance of microorganisms to an antimicrobial agent, in addition, to screen the diffusion of antimicrobial agent, the same bacteria species that tested in the of antibacterial activity were used in the MIC test. MIC test was done according to Krishnan *et al*. ( 2015).

**1-** First a stock of AgNPs 40 % was prepared by dissolved 720 mg of NPs powder in 18 ml of deionized water (the dissolve process was done using vortex and heating the solution in water bath 40 °C to make sure that all powder is dissolved completely).

**2-** Four different concentrations (20000,10000,5000,2500) µg/ml were prepared from the stock by serial dilution technique.

**3-** Each bacterium was compared with McFarland tube ($1.5x10^8$) CFU/ml . Then, cultured (100 µl) in a test tube contain 3ml of nanoparticles and Nutrient broth (N.B). As shown in figure (3-4).

**4-** The tubes were incubated at 37° C for 24 hours.

**5-** The MIC was determined based on the minimum concentration of AgNPs capable of preventing bacterial growth.

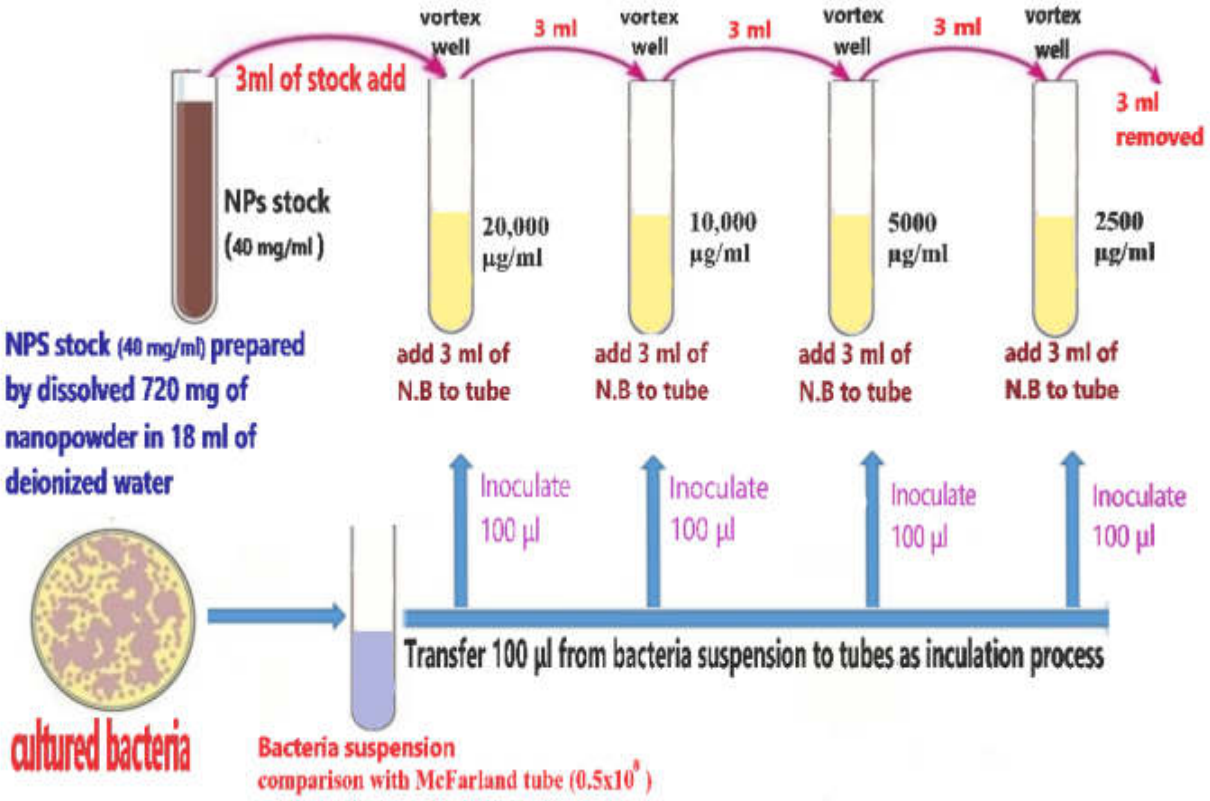



Figure (3-4) Determination the minimum inhibitory concentration (MIC) of Silver nanoparticles.

# Chapter four:
# Results and Discussion

# Results and Discussion

## 4.1 Isolation and identification of bacteria

A total of 200 clinical specimens were taken in this study. The wound was 100 swabs, the burns was 40 swabs while the urine was 60 samples. They cultured on selective and differential media. The bacterial species were identified after the initial diagnosis, from the total cultured swabs. The results showed that the isolation percentage of *Klebsiella pneumoniae* , *Escherichia coli* , *Proteus mirabilis* , *Pseudomonas aeruginosa* , *Staphylococcus aureus* and *Staphylococcus epidermidis* from wounds were 21(21%), 12(12%), 5(5%),15(15%),32(32%) and 15(15%), respectively; from the burns swabs were 14(35%),6(15%),0(0%), 7(17.5%), 10(25%), and 3(7.5%), respectively, and from urine were 18(30%),19(31.6%), 8(13.3%),15(25%),0(0%) and 0(0%), respectively, table (4-1).

**Table (4-1) Number and percentage of all bacterial isolates according to their sources.**

| Specimens | No.of speci-mens | *K. Pneumoniae* No.(%) | *E. coli* No.(%) | *P. mirabilis* No.(%) | *P. aeruginosa* No.(%) | *S. aureus* No.(%) | *S. epidermi dis* No.(%) |
|---|---|---|---|---|---|---|---|
| Wounds | 100 | 21(21) | 12(12) | 5(5) | 15(15) | 32(32) | 15(15) |
| Burns | 40 | 14(35) | 6(15) | 0(0%) | 7(17.5) | 10(25) | 3(7.5) |
| UTI | 60 | 18(30) | 19(31.6) | 8(13.3) | 15(25) | 0(0) | 0(0) |
| Total | 200 | 53(26.5) | 37(18.5) | 13(6.5) | 37(18.5) | 42(21) | 18(9) |

The results showed that the number of isolate *K. pneumoniae* from wound was 21(21%) which approximately agree with Al-Charrakh (2011) who reported its 24%. While from burns, it was 14 (35%) and from urine, it was 18 (30%) which approximately agree with AL-Zengena (2013) who reported them as 25% and 22%, respectively.

*Escherichia coli* isolation was 12(12%) from wounds which this agree with Ali *et al*. (2018), while from burns was 6(15%) and urine 19(31.6%), which matched with study in Kufa by Al-Salamy (2012) who reported *E.coli* percentage in urine was 44.4%.

*Proteus mirabilis* isolates were 5(5%) in wounds and UTI 8(13.3%) this matched with (Hussein, 2013).

*Pseudomonas aeruginosa* isolation was 15(15%),7(17.5%) and 15(25%) from wounds, burns and urine, respectively. This approximately agree with Al-Imari (2011) who reported that they were 12.2% ,14.3%, 22.4%. *S. aureus* isolation was 32(32%) and 10(25%) from wounds and burns, respectively. This matched with another study in the same location by (Sahm, 2019). *S. epidermidis* isolation were 15(15%) and 3(7.5%) from wounds and burns respectively.

The variation in isolation percentages between the studies may be attributed to many factors, such as geographical location, health awareness of individuals, number of samples studied, season of sampling, sanitary practices in hospitals and staff, environmental conditions, isolation and identification techniques, social and cultural level of patients. All these factors may play an important role in inhibition or stimulation of the growth and distribution of pathogenic bacteria in the hospitals.

### 4.1.1 Initial Identification of Isolated Bacteria

*Escherichia coli , Pseudomonas aeruginosa , Staphylococcus aureus* and *Staphylococcus epidermidis* were the only bacterial isolates that subculture on selective and differential media.

*Escherichia coli* isolates were subculture on Eosin methylene blue (EMB) agar medium. *E. coli* was appeared Green metallic sheen. Rapid fermentation of lactose & production of strong acids. Thus, a rapid reduction in the pH of the EMB agar and formation of the green metallic sheen were observed with *E. coli*.

*Pseudomonas aeruginosa* isolates were subculture on Pseudomonas agar medium. Pseudomonas agar medium is recommended for use in the qualitative procedures for differentiation *P. aeruginosa* from other *Pseudomonas* spp. *Pseudomonas aeruginosa was* appeared deep green color.

*Staphylococcus aureus* and *Staphylococcus epidermidis* isolates were subculture on mannitol salt agar medium (MSA) which was utilized as a specific media for Staphylococci and Micrococci and for separation of *S. aureus* from *S. epidermidis (*Macfaddin, 2000*). S. aureus* colonies was appeared as golden color with yellow zones while *S. epidermidis* was small pink or red colonies with no color change to the medium.

### 4.1.2 Identification by Biochemical tests

Several biochemical tests were performed to confirm the diagnosis of bacterial isolates. All the presumptive isolates of *P. aeruginosa* showed a positive result for oxidase and catalase utilizing citrate as carbon source. They showed negative result for indol, methyl red and vogas-proskauer , urease, Kligler's iron and growth on Pseudomonas agar.

All the preliminary diagnosis isolates of *P. mirabilis* showed a positive result for catalase, methyl red, citrate utilization, urease and Kligler's iron ($K/A^{-}$ $^{+}$ $H_2S$ produce), but they were negative result for oxidase, indole and vogas-Proskauer.

The isolates of *E. coli* were positive for catalase, indole, methyl red and Kligler's iron ($A/A^{++}$), but they were negative for oxidase, vogas-Proskauer, citrate utilization and urease.

Presumptive isolates of *K. pneumoniae* appeared as a positive for catalase, vogas-Proskauer, citrate utilization, Kligler's iron($A/A^{++}$) and Urease, but they were negative for oxidase and indole. These results were explained in table (4-2) and figures (1-1) , (1-2) , (1-3) and (1-4) in appendix.

Staphylococci were identified depending on the cultural and the microscopical properties as well as biochemical tests. The isolates of *S. aureus* and *S. epidermidis* were a positive for catalase, but they were negative for oxidase table (4-3).

**Table (4-2) Identification of gram negative bacteria by biochemical tests**

| Type of isolate / Tests | *Proteus mirabilis* | *Pseudomonas aeruginosa* | *Escherichia coli* | *Klebsiella pneumoniae* |
|---|---|---|---|---|
| **Catalase** | (+ve) | (+ve) | (+ve) | (+ve) |
| **Oxidase** | (-ve) | (+ve) | (-ve) | (-ve) |
| **Indole** | (-ve) | (-ve) | (+ve) | (-ve) |
| **Methyl red** | (+ve) | (-ve) | (+ve) | (-ve) |
| **Vogas-Proskauer** | (-ve) | (-ve) | (-ve) | (+ve) |
| **Citrate utilization** | (+ve) | (+ve) | (-ve) | (+ve) |

| | | | | |
|---|---|---|---|---|
| **Kligler's iron** | K/A $H_2S$ produce | K/K -- | A/A ++ | A/A ++ |
| **Urease production** | (+ve) | (-ve) | (-ve) | (+ve) |
| Gram stain | Gram-negative (pink) rod-shaped bacterium | Gram-negative (pink) rod-shaped bacterium | Gram-negative (pink ) rod-shaped bacterium | Gram-negative (pink ) rod-shaped bacterium |
| **(+ve) : Positive result**<br>**(- ve) : Negative result** | | | K:Alkaline<br>A:Acid | |

**Table (4-3): Identification of gram positive bacteria *Staphylococcus* spp. by biochemical tests.**

| **Biochemical tests** | **Result** | |
|---|---|---|
| | *S. aureus* | *S. epidermidis* |
| **Mannitol salt agar** | Yellow colonies | Pink colonies without changing the color of the medium |
| **Gram stain** | Gram positive cocci | Gram positive cocci |
| **Oxidase test** | (-) | (-) |
| **Catalase test** | (+) | (+) |
| ➢ **(+): Positive result**<br>➢ **( -): Negative result** | | |

### 4.1.3 Identification of isolated bacteria by VITEK-2 system

The most antibiotics resistant isolates were selected and confirmed the identification by VITEK-2 system appendix (1-5),(1-6),(1-7),(1-8),(1-9),(1-10).

## 4.2 Antimicrobial susceptibility test

All different bacterial species were tested toward 29 antimicrobial agents using disk diffusion method. The results were interpreted according to the recommendation of CLSI (2019) and showed that there were many bacteria isolates of multiple drugs resist (MDR) from different types of bacteria.

### 4.2.1 Antimicrobial susceptibility test of *Staphylococcus aureus* and *Staphylococcus epidermidis*

The results of statistical analysis of total (42) isolates of *S. aureus* showed a varied levels of resistances to Ciprofloxacin 17(40%), Ofloxacin 16(38%), Levofloxacin 25(59.5%), Azithromycin 32(76%), Clarithromycin 34(80%) ,Tetracycline 27(64.2%), Doxycycline 17(40.4%), Clindamycin 13(30.9%), Streptomycin 16(38%), Gentamicin18(42.8%), Vancomycin 12(28.5%) and Chloramphenicol 8(19%).

*Staphylococcus aureus* showed high percent of resistance towards the following antimicrobial agents; Clarithromycin 80%, Azithromycin76%, Tetracycline 64.2%, Levofloxacin 59.5%, Gentamicin42.8%, Doxycycline 40.4% and Streptomycin 38%.

The results of resistance to antibiotics Azithromycin and Vancomycin correspond with the study of Al-Tameemi (2012) who showed that the resistant to these antibiotics were 78% and 30% respectively, but disagree with the results obtained by Sahm (2019) who showed the resistant to these antibiotics were 92% and 10%, respectively.

Vancomycin is a good choice for treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Tiwari, 2007).

The first appear of vancomycin resistant *S. aureus* strain was in Japan in 1997, the level of vancomycin-resistant *Staphylococcus aureus* (VRSA) began to increase that increasing health risk .The widespread and excessive use of glycopeptides antagonists, such as vancomycin and teicoplanin to treat *staphylococcus* infections in humans, has led to the emergence of VRSA strains. There are six genes responsible for vancomycin resistance; *van* A, *van* B, *van* C, *van* D, *van* E, *van* G. The *van* A is the most common. The *van* A genotype was classified as the common among VRSA strains and based on sensitivity to teicoplanin. The *van* A gene phenotype was classified based on resistance to both vancomycin and teicoplanin. The *van* B gene, that came in second place, was characterized by resistance to vancomycin but sensitive to teicoplanin (Nicho *et al*., 2006).

In this study, the resistance to tetracycline was 64.2% which agreed with result obtained in another local study of Zeidan (2005) who showed that the rates of resistant to tetracycline was 60%, while this results disagree with those obtained by another local study of Al-Geobory, (2011).

*Staphylococci* are resistant to tetracycline, by two mechanisms: first, active efflux of the antibiotic; second, ribosomal protection mediated by *tet*M or *tet*O determinants located on either a transposon or the chromosome. The *tet*M gene, in contrast, is believed to confer resistance to all drugs of the tetracycline group, including tetracycline and minocycline. *Tet* K is the major gene in *Staphylococci* that encoding active efflux (Strommenger *et al*., 2004). The rest of the antibiotics that our tested bacteria resisted did not agree with the previous studies due to the difference in

isolation areas, types of antibiotics and time period between the previous studies and the current study.

Eighteen isolates of *S. epidermidis* were resistant to the following antibiotics: Ciprofloxacin 6 (33.3%), Ofloxacin 5(27.7%), Levofloxacin 8(44.4%), Azithromycin 12(66.6%), Clarithromycin 12(66.6%), Tetracycline 5(27.7%), Doxycycline 4(22.2%), Clindamycin 2(11%), Streptomycin 3(16.6%), Gentamicin 8(44.4%), Vancomycin 2(11%) and Chloramphenicol 2(11%). The figure (4-1) Show the antibiotic resistance of *S. aureus* and *S. epidermidis.*

*S. epidermidis* showed high percent of resistance towards the following antimicrobial agents; Azithromycin and clarithromycin (66.6%), gentamicin and levofloxacin (44.4%).

In this study *S. epidermidis* were resistant to ciprofloxacin, gentamycin and tetracycline. These results were similar to that obtained by Fadhel (2013) who reported 50% were resistant to both ciprofloxacin and gentamycin and 20% to tetracycline. The rest of the antibiotic resistance of the bacteria did not match the previous studies due to the difference in isolation areas, types of antibiotics and time period between the previous studies and the current study.

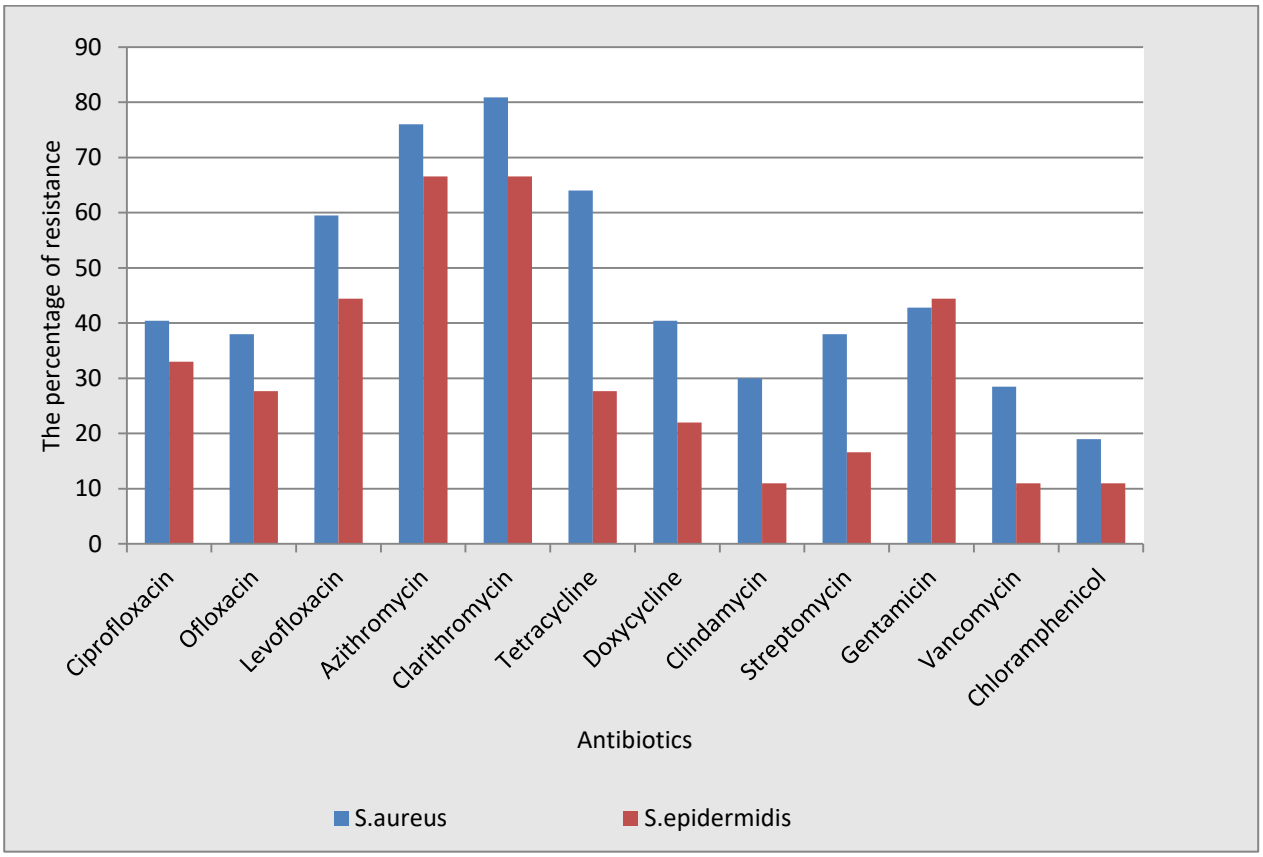



**Figure (4-1) The Antibiotic Resistance of *S. aureus* and *S. epidermidis***

**Table (4-4) Number and percentage of MDR,XDR and PDR of *S. aureus* and *S. epidermidis***

| Bacteria | No and (%) of MDR | No and (%) of XDR | No and (%) of PDR |
|---|---|---|---|
| ***S. aureus*** | 17(40.47%) | 2(4.74%) | 0(0%) |
| ***S. epidermidis*** | 4(22.2%) | 0(0%) | 0(0%) |

### 4.2.2 Antimicrobial susceptibility test of *Klebsiella pneumoniae* and *Proteus mirabilis*

The results of statistical analysis showed 53 isolates of *K. pneumoniae* and 13 isolates of *P. mirabilis* expressed a varied levels of resistances to antibiotics (figure 4-2). For *K. pneumoniae,* the resistance percentage were as following: Ciprofloxacin 28(52.8%), Ofloxacin 26(49%), Gentamicin 27(51%), Trimethoprim 40(75.4%), Cefotaxime 39(73.5%), Cefuroxime 30(56.6%), Amikacin 10(18.8%),Streptomycin 33(62.2%), Nitrofurantoin 20(37.7%),Piperacillin 35(66.03%),Imipenem 20(37.7%) and Chloramphenicol 13(24.5%).

*Klebsiella pneumoniae* showed the highest resistance to the following antimicrobial agents; Trimethoprim 75.4%, Cefotaxime 73.5%, Piperacillin 66.03%, Streptomycin 62.2%, Cefuroxime 56.6% and Ciprofloxacin 52.8 %.

Bacterial resistance to Piperacillin, Amikacin, and Trimethoprim were approximately agree with the result obtained in another local study of AL-Zengena, (2013) who reported resistance to the antibiotics were 95%, 19% and 70%, respectively.

*Klebsiella pneumoniae* possesses a large plasmid that confers resistance to Cefotaxime. Resistance to Cephalosporins, especially the third generation, may occur as a result of the bacterial overproduction of the chromosomal enzyme cephalosporinase, which belongs to group β-lactamases. The resistance of bacterial isolates to aminoclycosides(Gentamicin, Amikacin and Streptomycin)indicates that the isolates have a high-level resistance to this group (Li and Lim, 2000).

The resistances of *Klebsiella pneumoniae* to Ciprofloxacin is mediated by flow pumps that cause multiple antibiotic resistance and are regulated by 38 genes or

operons in the bacteria. Nineteen of these genes are directly responsible for developing resistance to antibiotics, including quinolones, by altering the target molecule and reducing cellular permeability (Yedekci *et al*., 2012).

*Proteus mirabilis* resistance to tested antibiotics were as follow: Ciprofloxacin 1(7.6%), Ofloxacin 1(7.6%), Gentamicin 4(30.7%), Trimethoprim 8(61.5%), Cefotaxime 7(53.8%), Cefuroxime 7(53.84%), Amikacin 3(23%), Streptomycin 10(76.9%), Nitrofurantoin 6(46.15%), Piperacillin 4(30.7%), Imipenem 0(0%) and Chloramphenicol 5(38.46%).

The highest resistance to antimicrobial agents were: Streptomycin 76.9%, Trimethoprim 61.5% , Cefuroxime53.8% and Nitrofurantoin 46.15%.These results agree with result obtained in local study done by Hassan (2018).

The intrinsic resistance of *Proteus* spp. to nitrofurantoin is acquired chromosomally (mutations) or extra chromosomally mediated (plasmids) (Reynard *et al*., 2013).

Some *Proteus mirabilis* showed an elevation in the resistance level to imipenem due to many reasons, including; the loss of outer membrane porins, decreasing expression of penicillin-binding protein (PBP1a) or reducing binding of imipenem by PBP2 (Girlich *et al*., 2014). The present study is mismatching with the result of Pal *et al*. (2014) who demonstrated 6% of *P. mirabilis* were resistant to imipenem.

The low rate of resistance to Ciprofloxacin and Amikacin were in agreement with the results obtained by Al-Azzawi (2018) who reported it as; 1% and 15%, respectively.

Our study showed that the isolates were resistant to Gentamicin which closed to the result obtained in another study done by Al-jeboury (2005) who reported that the resistance to gentamicin was 30%. However, the result of this study disagreed with

Hassan (2018) who reported it as 54%.The genetic studies indicated that the resistance genes of Gentamycin are present on mobile genetics element that can facilitate their horizontal gene transfer and that genes are found on R plasmid of the Enterobacteriaceae (Diaz *et al*., 2006). The figure (4-2) Show the antibiotic resistance of *K. Pneumoniae* and *P. mirabilis.*

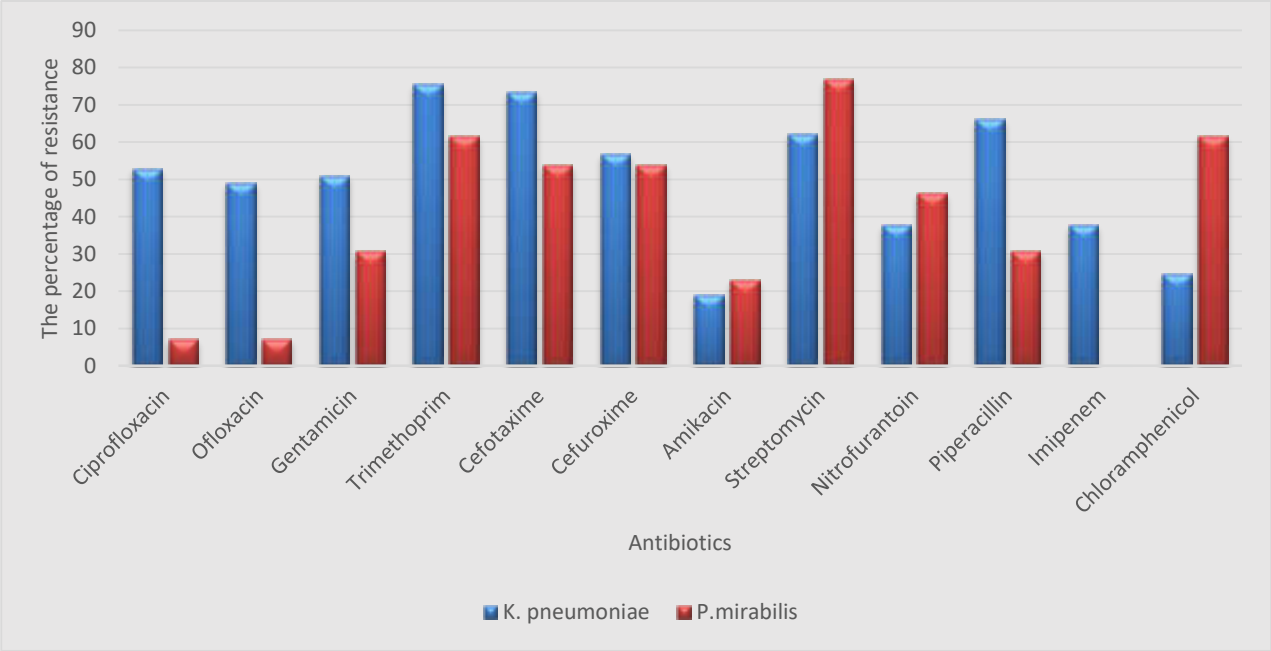



**Figure (4-2) Antibiotic resistance of *K. pneumoniae* and *P. mirabilis.***

**Table (4-5) Number and percentage of MDR,XDR and PDR of *K. pneumoniae* and *P. mirabilis.***

| Bacteria | No. and (%) of MDR | No. and (%) of XDR | No. and (%) of PDR |
|---|---|---|---|
| ***K. pneumoniae*** | 24(56.6%) | 3(5.6%) | 2(3.77%) |
| ***P. mirabilis*** | 7(53%) | 0(0%) | 0(0%) |

### 4.2.3 Antimicrobial susceptibility test of *Escherichia coli*

In our results, the total 37 isolates of *E. coli* showed a varied levels of resistances to the following antibiotics: Imipenem 22(59.4%), Tetracycline 24(64.8%), Doxycycline 27(72.9%),Aztreonam29(78.3%),Levofloxacin23(62%),Cefpodoxime33 (89%),Trimethoprim sulfamethoxazole 31(83.7%), Azithromycin29(78.3%), Ampicillin-sulbactam13(35%),Ticarcillin-clavulanate20(54%),Cefuroxime29(78.3%) and Cefoxitin 21(56.7%),(figure 4-3).

*Escherichia coli* showed the highest resistance to antimicrobial agents including; Cefpodoxime (89%), Trimethoprim-Sulfamethoxazole (83.7%), Aztreonam(78.3%), Azithromycin and Cefuroxime(78.3%).

The bacteria resist β-lactam by producing beta-lactamase enzymes, which are an important way to resist beta-lactam drug. Most genera of Gram-negative bacteria have genes that encode β-Lactamase enzymes, one is chromosomal and the other is a plasmid which could transmit resistance gene among the strains of the *Enterobacteriaceae* family(Turner, 2005). These enzymes in Gram-positive and negative bacteria break down the amide bond of the beta-lactam ring. This turns penicillins into pencilloic acid and cephalosporins to Cephalosporonic acid. As a result, these antimicrobial become ineffective and lose their ability to bind to the Penicillin-binding proteins (PBPs).

The resistance of levofloxacin were 62% was approximately agree with local study done by(Salman, 2013) who reported it as 55%. The resistant of other antibiotics by bacteria not match the previous studies due to the difference in isolation areas, types of antibiotics and time period between the previous studies and this study.

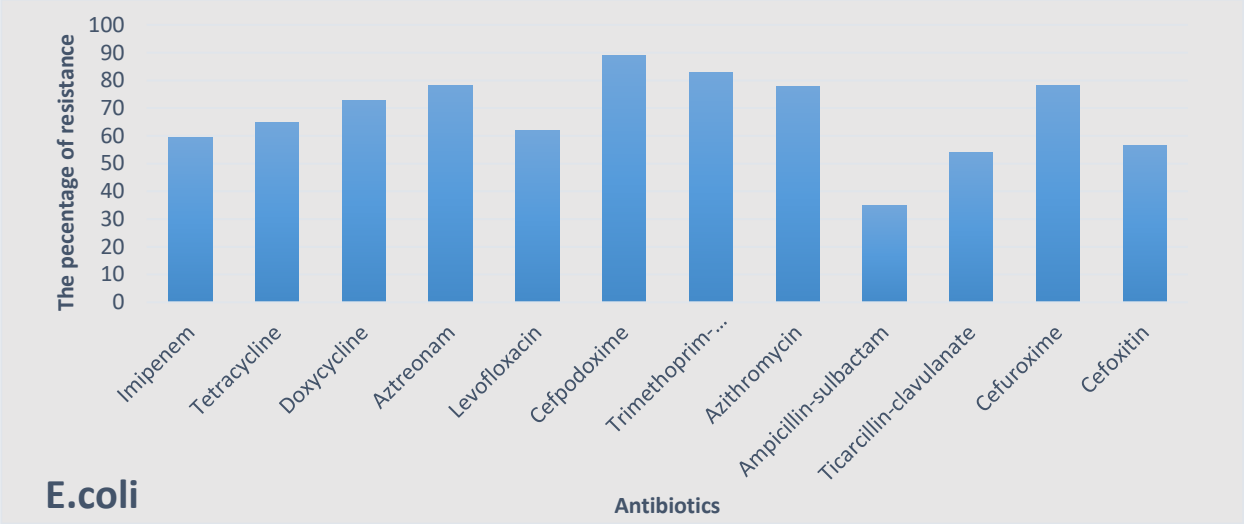



**Figure (4-3) Antibiotic resistance of *E. coli***

**Table (4-6) Number and percentage of MDR,XDR and PDR of *E. coli***

| Bacteria | No and (%) of MDR | No and (%) of XDR | No and (%) of PDR |
|---|---|---|---|
| ***E. coli*** | 19(51.35%) | 3(8.1%) | 0(0%) |

## 4.2.4 Antimicrobial susceptibility test of *Pseudomonas aeruginosa*

In this study, the results of the total 37 isolates of *P. aeruginosa* showed a varied levels of resistances to the following antibiotics: Ciprofloxacin 2(5.4%), Ofloxacin 8(21.6%), Gentamicin 11(29.7%), Tobramycin 8(21.6%), Aztreonam 15(40.5%), Levofloxacin4(10.8%), Amikacin 10(27%),Meropenem 6(16%),Ceftazidime 10(27%), Piperacillin 15(40.5%) , Imipenem 2(5.4%),Cefepime 13(35%), (figure4-4).

*P. aeruginosa* showed the highest resistance to antibiotics Piperacillin, Aztreonam and Cefepime.

The results appeared that the percentage of bacterial resistant to Gentamicin was 30% and Amikacin 27%. These findings were agree with another local study done by Abbas (2018) who reported 34% and 30%, respectively. Also the low resistant to

Levofloxacin (10.8%) Meropenem (16%) approximately was closed to the same study which was reported them as 5% and 10%, respectively.

The resistance to Cefepime 35% , Ceftazidime 27%, Imipenem and Ciprofloxacin 5.4% were agree with another local study done by Al-Kubaisy (2018) who reported them as 25%,15%,2% and10%, respectively. These results were mismatched in another local study by Rubaye (2019) who reported them as 78%,51%,49.5% and 56.5%, respectively.

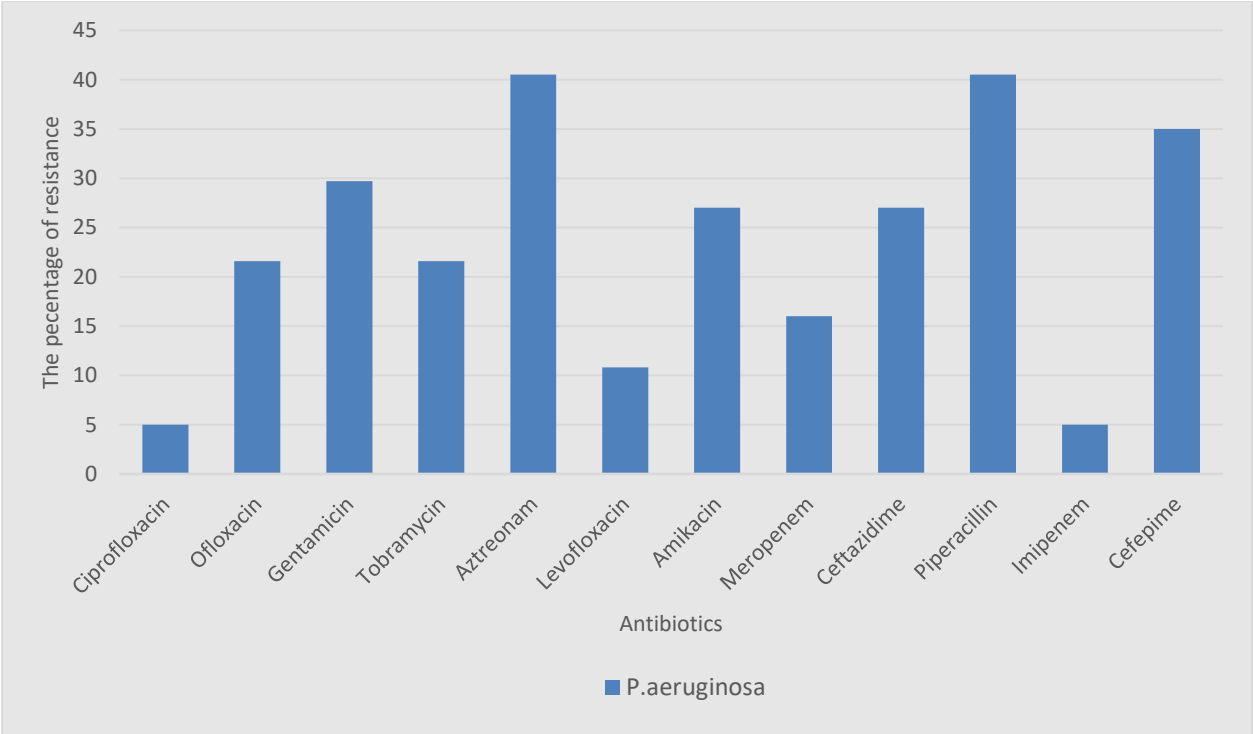



**Figure (4-4) Antibiotic Resistance of *P. aeruginosa***

**Table (4-7) Number and percentage of MDR,XDR and PDR of *P. aeruginosa***

| Bacteria | No and (%) of MDR | No and (%) of XDR | No and (%) of PDR |
|---|---|---|---|
| ***P. aeruginosa*** | 11(29.7%) | 0(0%) | 0(0%) |

From each one of these bacterial isolates, one of MDR isolate , were selected to complete the current study. The selection was based on the basis of most resistant bacteria to antibiotics. The tables (1-1), (1-2), (1-3), (1-3), (1-5) , (1- 6) showed in the appendix the bacterial species that selected, their sensitivity to the tested antibiotics and their sources.

## 4.3 Isolation and identification of *Lactobacillus* spp.

Ten samples of fermented milk products (provincial dairy products) were primarily grown onto MRS agar plates (by serial dilution technique), as selective media for isolation, and incubated at 37 °C for 24- 48 hr under micro-aerobic condition using candle jar. The colonies surrounded by clear zone due to the hydrolysis of calcium carbonate in the medium by the acid that produced from the fermentation of the lactose sugar **.**

### 4.3.1 Macroscopic and Microscopic Morphology of *Lactobacillus* spp.

The colony of *Lactobacillus* was identified as a small (2-5 mm), convex, smooth, glistening colony, and opaque without pigment (Holt *et al*., 1994). Microscopically, the bacteria appeared under oil immersion as Gram positive bacilli, arranged in singly pairs or short chains.

### 4.3.2 Identification of *Lactobacillus* spp. by Biochemical tests

The isolates that confirmed belong to genus *Lactobacillus* was identified depending on the using of biochemical tests. Results revealed that all isolates were negative to oxidase, catalase, indole tests. They were capable of hemolysin production on blood agar medium which indicated α- hemolysis (Holt *et al*.,1994), (table 4-8).

**Table(4-8) Identification of *Lactobacillus* isolates by biochemical tests**

| Biochemical Test | Results |
|---|---|
| **Gram stain** | + |
| **Oxidase** | - |
| **Catalase** | - |
| **Indole production** | - |
| **Growth on MRS** | + |
| **Hemolysis** | α- hemolysis |

## 4.4 Biosynthesis of silver nanoparticles by *Lactobacillus* spp.

*Lactobacillus* spp. were screened for production of (AgNPs) using their extracellular (cell free supernatant) "CFS" of bacterial cultures in skim milk (pH 5 and 37 °C). The primary sight for AgNPs formation was change of color of reaction mixture from pale yellow to dark brown after adding $AgNo_3$ . This change in color could be noticed by the nicked eye. As the color intensity increased, the accumulation of AgNPs increased (Elbeshehy *et al*., 2015), as appeared in figure (4-5). The control; CFS (whey) without silver nitrate showed no color formation when incubated for the same period and condition. After that, the possible biosynthesis of AgNPs was harvested in powder form.

The knowledge about the reduction of silver ions and formation of silver nanoparticles were still not clear, but it is believed that the protein molecules and enzyme, includes nitrate reductase enzyme act as good regulating agent in silver nanoparticles synthesis (Wang *et al*., 2017).

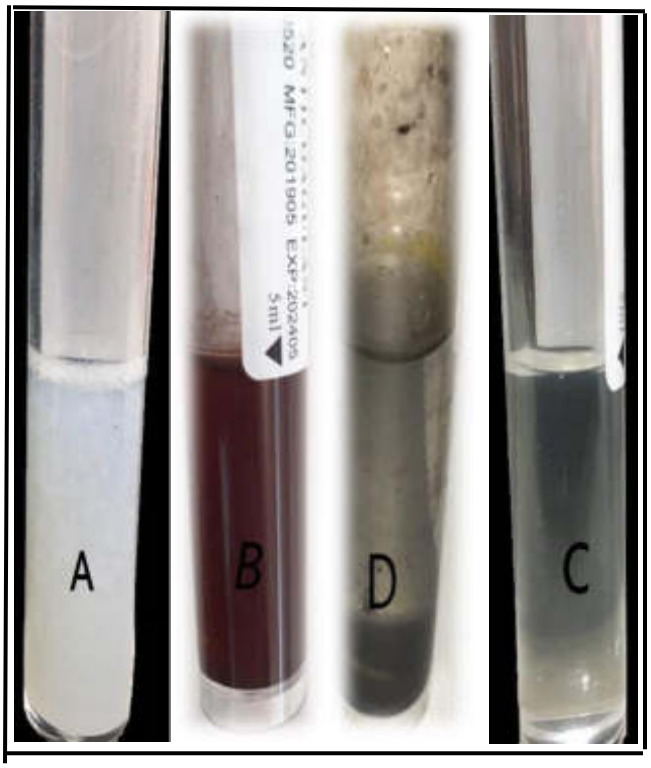



Figure (4-5) Screening of *Lactobacillus* spp. for silver nanoparticle biosynthesis:

(C) The CFS(whey) without $AgNo_3$ (control).

(A) The moment of adding silver nitrate to the CFS.

(B)The color of the CFS change to deep brown after 6 hours form adding $AgNo_3$.

(D) The precipitate formed at the bottom of the tube after 24 hours from the addition of silver nitrate.

## 4.5 Characterization of Silver Nanoparticles

### 4.5.1 X-Ray Diffraction (XRD) analysis

Pattern of X-Ray Diffraction measurements are used to examine structure intersystem of crystalline behavior of films at special condition. Nanoparticles were characterized using X-ray diffractometer (Bruker D8, Karlsruhe, Germany).The incident and reflected angles are scanned with following specifications: target is Cu,

Wavelength is 1.5406 A˚, current is 30 mA and voltage is 40 KV. The incident and reflected angles are scanned using 2θ technique. XRD was conducted to investigate the presence of nanomaterials and diagnose them. The results as shown in figure (4-6) and in appendix figure (1-11) was determined the presence of two types of nanomaterials Ag and Agcl in the sample, these results are consistent with the results ( Zhao *et al*., 2015). As shown in figure (4-6) the characteristic XRD peaks at 38.1°, 44.2°,64.4° and 77.4° were attributed to (111), (200), (220) and (311) reflection planes for cubic Ag (JCPDS No. 4-783). The clear and dominant five peaks at 27.8°, 32.2°, 46.2°, 54.8°, and 57.4 were attributed to planes (111), (200), (220), (311), (222) cubic phase of AgCl crystal (JCPDS No. 31-1238). No other characteristic peaks could be attributed to impurities which indicated the high purity of Ag/AgCl. The strong and narrow diffraction peaks revealed the highly crystalline (Wang *et al*., 2010).

The particle size of Ag and AgCl is calculated by Scherrer equation {d = Kλ/βcosθ}, d is the mean size of the ordered (crystalline) domains; K is a dimensionless shape factor, with a value close to unity. The shape factor has a typical value of about 0.91; **λ** is the X-ray wavelength; β is the line broadening at half the maximum intensity (FWHM), after subtracting the instrumental line broadening, in radians{ $\beta_{(radian)} = 2\pi\ \beta_{(deg.)} /360$}.θ is the Bragg angle of the main crystal plane in radians{ $\theta_{(radian)} = 2\pi\ \theta_{(deg.)} /360$}. We chose the three strongest peaks (38.1) and (77.3) of Ag and (32.2) of AgCl to calculate the particle size. The calculated particle size was 17.1and 18.6 nm for Ag respectively, and 24.3 nm for AgCl.

The producing process of silver chloride nanoparticles occurred through one of two possibilities according to the inorganic chemistry of silver (Trinh *et al*., 2015):

1)During the interaction of the silver nitrate solution with the CFS (whey), we assumed that the silver chloride nanoparticles (AgCl-NPs) were formed immediately as a result of the exchange reaction of silver nitrate with trace (NaCl) in the whey.

$$\mathbf{AgNO_3(s) + whey_{(aq)}\,(NaCl) \rightarrow NaNO_3(s) + AgCl(s)}$$

2) During the using of HCl solution to adjust pH (5.5-6) of MRS medium to make the medium perfect for growth of *Lactobacillus* spp., and during harvesting the whey, small amounts of HCl were present within it.

$$\mathbf{HCl_{(aq)} + AgNO_3(s) \rightarrow AgCl(s) + HNO_3(s)}$$

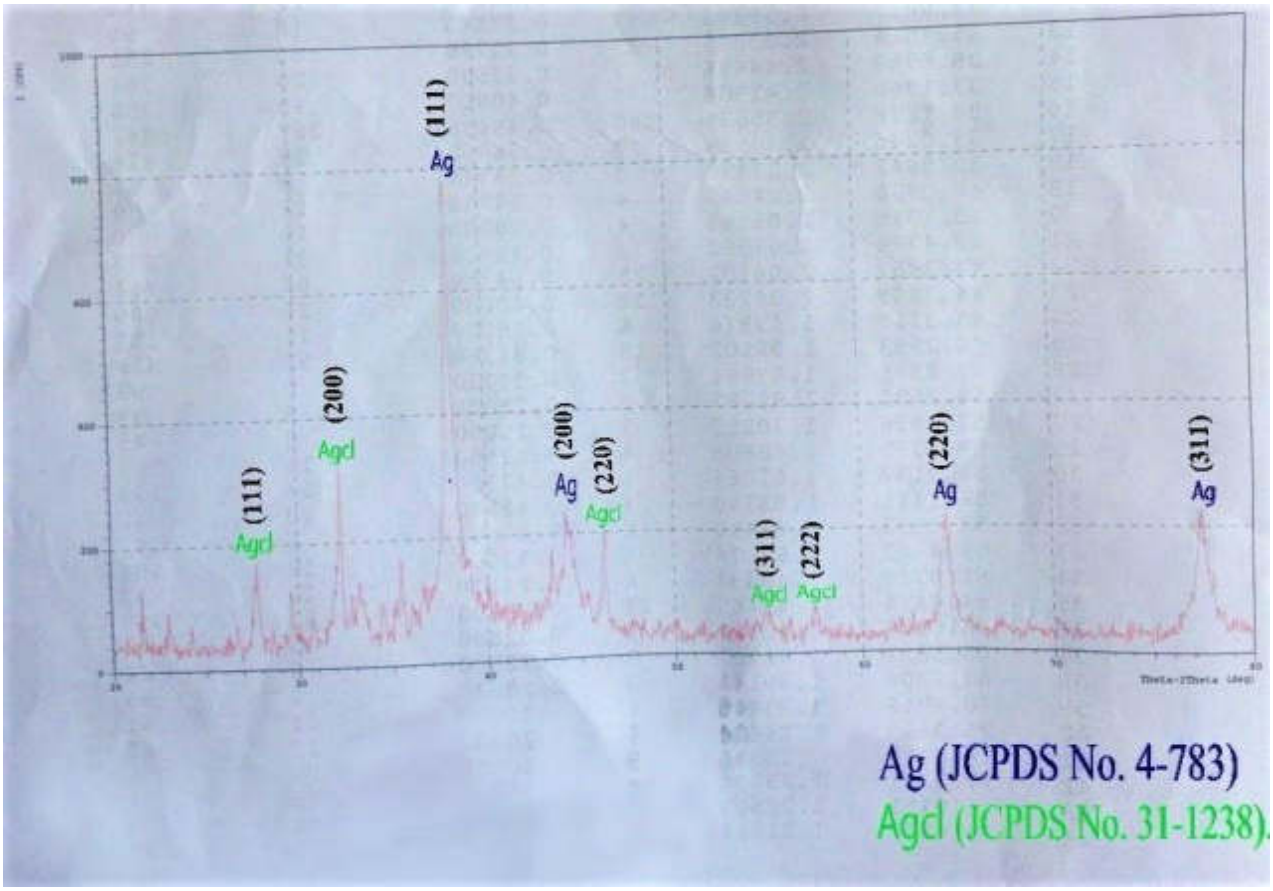



Figure (4-6) X-ray Diffraction (XRD) analysis

## 4.5.2 Atomic Force Microscopy(AFM)

The size, topography and granularity volume distribution of biosynthesized nanoparticles that are characterized by the use Atomic Force Microscopy (AFM).Table (4-9) indicated that Ag NPs formed by *Lactobacillus* spp. was in average diameter 50 nm. The presence of bright spots on the surface as found in AFM is indicating the presence of Ag/AgCl-NPs, and the inverse showed the dark spots indicating size and dispersion of the nanoparticles. The images from AFM showed rotational ellipsoidal and cubic shapes of nanoparticles, figure (4-7).

Table (4-9) The Cumulation size of Ag/Agcl biosynthesis by *Lactobacillus* spp.

| **Avg. Diameter:50 nm** | | | | | **Diameter:35.00 nm ≤10%** | | | |
|---|---|---|---|---|---|---|---|---|
| **Diameter:50.00 nm≤50%** | | | | | **Diameter:55.00 nm≤90%** | | | |
| **Diameter (nm)<** | **Volume( %)** | **Cumulatio n(%)** | **Diameter (nm)<** | **Volume( %)** | **Cumulatio n(%)** | **Diameter (nm)<** | **Volume( %)** | **Cumulat -ion(%)** |
| **35.00** | **1.73** | **1.73** | **50.00** | **20.00** | **45.35** | **65.00** | **9.13** | **100.00** |
| **40.00** | **10.24** | **11.97** | **55.00** | **25.51** | **70.87** | | | |
| **45.00** | **13.39** | **25.35** | **60.00** | **20.00** | **90.87** | | | |

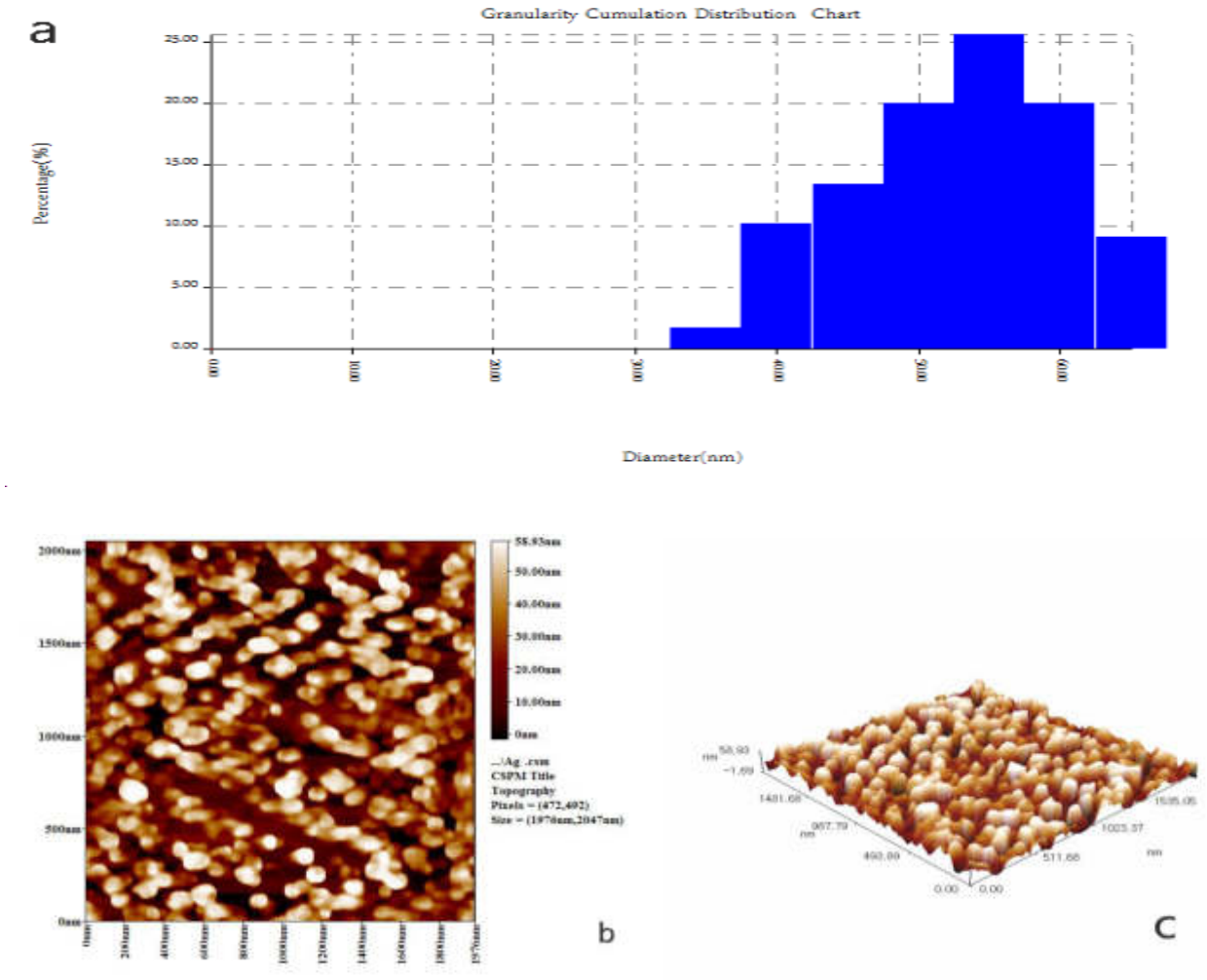



Figure (4-7) Biosynthesized Ag/AgCl NPs by of *Lactobacillus* spp.

(a) Granularity Distribution Chart of silver nanoparticles synthesis

(b) 2D image of Silver Nanoparticles synthesis

(c) 3D image of Silver Nanoparticles synthesis

### 4.5.3 Scanning Electron Microscopy (SEM) analysis

A scanning electron microscopy (SEM) is a sort of electron magnifying lens that produce pictures of a sample by examining it with an engaged beam of electron. The electrons interface with particles in the specimen, creating different signs that can be

distinguished and that contain data about the samples surface geology and organization. Scanning Electron Microscope (SEM) image of Ag/AgCl NPs sample .The results showed that size of Ag/AgCl as following: (23.15, 24.77 , 25.14 , 27.07, 27.35, 29.23, 31.18, 31.24, 31.56, 31.8, 32.19 ,32.41 ,33.8 ,35.8, 36.25, 38.15, 38.66, 39.26, 41.63, 42.95, 44.11, 51.76, 53.98 and 57.37nm) average size was 35.8 nm, as clear in figure (4-8).

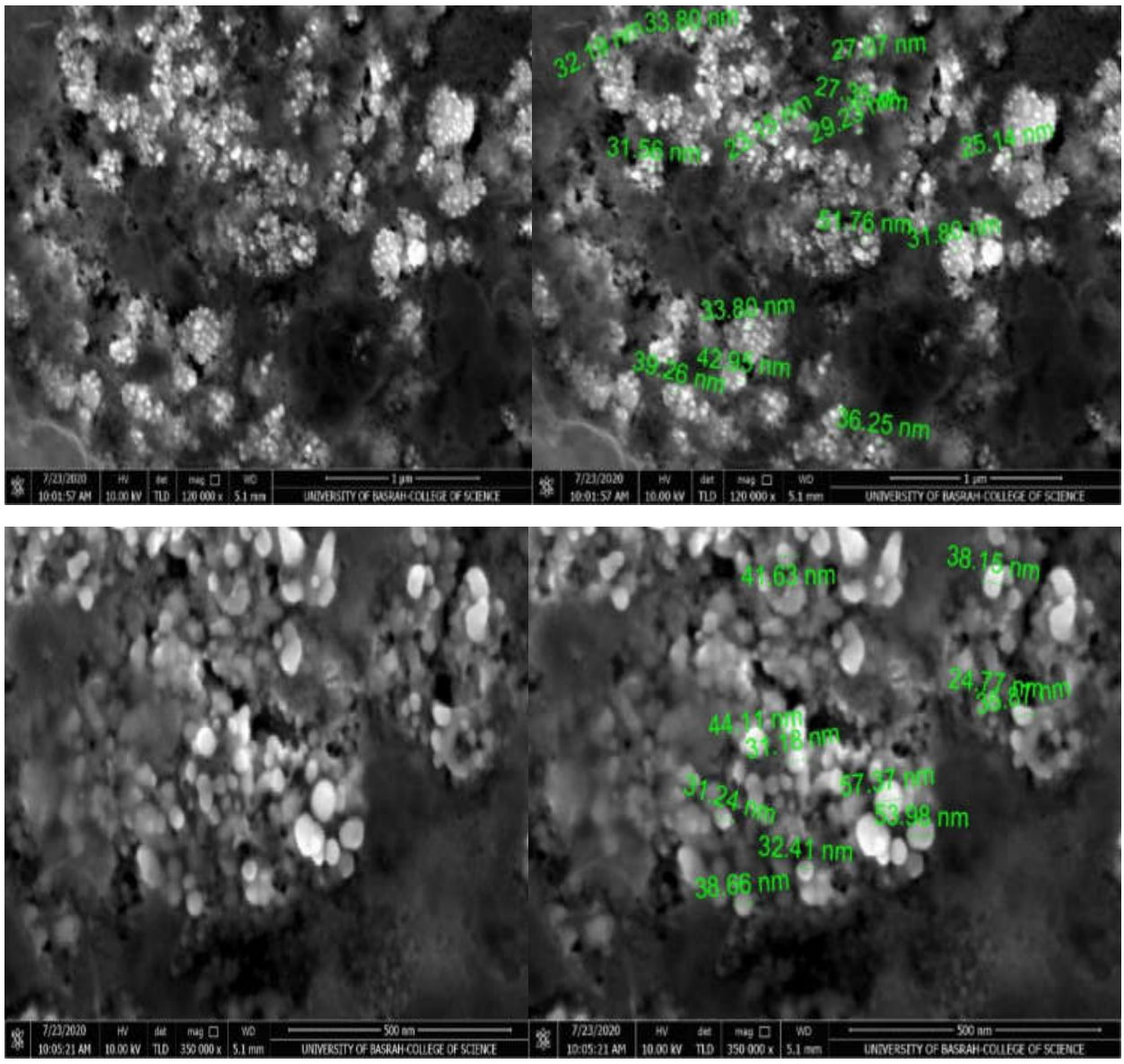



Figure (4-8) The SEM images of Ag/AgCl nanoparticles

### 4.5.4 UV-Vis Spectroscopy

Different molecules can absorb ultra-violate and visible light at different wave lengths. This assay estimated the intensity of absorption or optical absorption of solution in order to get the particles formation peak. The corresponding UV-Vis absorption spectra are shown in figure (4-9). UV-visible spectra of synthesized AgNPs were recorded in the range of 320–500 nm (Litvin *et al*.,2013). According to Vilamová *et al*. (2019) UV-Vis Spectroscopy confirmed the characteristic absorption peaks at 460 nm for AgCl NPs.

As explained by Nayak *et al*. (2011), the biological method of synthesis of silver nanoparticles exhibit strong absorption of electromagnetic waves in the visible range due to their optical resonant property, called Surface Plasmon Resonance (SPR). The SPR is highly influenced by the shape and size of the nanoparticles. Due to the small size of silver nanoparticles in the quantum regime, the sample exhibited a stronger absorption at (400–650 nm). The strong absorption of visible light was responsible for visible-light-driven photocatalytic activity of Ag/AgCl. The absorption band between (290-350 nm) could be attributed to the characteristic absorption of AgCl composite, which possesses a direct band gap of 5.6 eV and an indirect band gap of 3.25 eV. This findings were matched with the study of Zhao *et al*. (2015). The narrow range was indicating the similarity of particles size and absence of particles aggregation. The AgNPs “absorb radiation intensity” at wavelength of 400 nm because of the transition of electrons . Kushwaha *et al.*, (2015) observed that the turbidity of silver nanoparticles which were biosynthesized by *E. coli* was measured at 400nm. Another study by Deljou and Goudarzi (2016) showed that silver nanoparticles biosynthesized by thermophilic *bacillus* spp. was at absorbance 425nm.

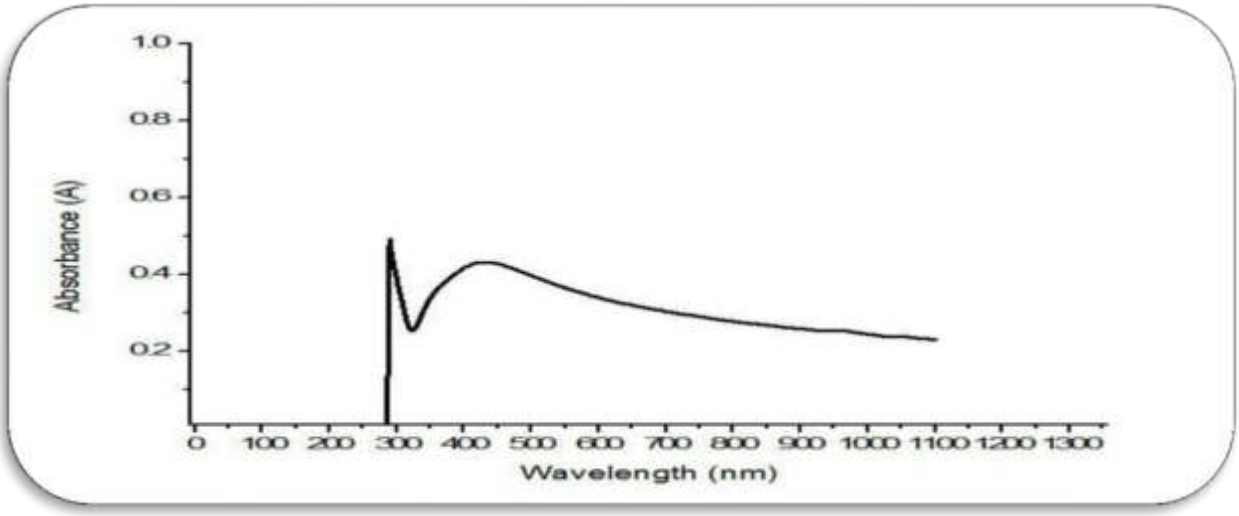



Figure (4-9) UV-visible spectroscopy of synthesized Ag/AgCl NPs.

### 4.5.5 Fourier-Transform Infrared Spectroscopy (FTIR) analysis

FTIR was performed to determine the possible functional groups of biomolecules involved in the reduction of silver and silver chloride ions and stabilization of the biosynthesized Ag/AgCl NPs from *Lactobacillus* spp. FTIR analysis of the dried powder of Ag/AgCl NPs was carried out by scanning the spectrum in the range 400–4000 $cm^{-1}$ at a resolution of 4$cm^{-1}$ (8400S/Shimadzu / Japan). The FTIR spectrum of biosynthesized Ag/AgCl NPs showed eight distinct peaks; 416, 540, 1031, 1382, 1600, 2366, 2927 and 3381 $cm^{-1}$ as shown in the figure (4-10). The peak at 3381 $cm^{-1}$ corresponded to asymmetrical and symmetrical N−H stretching vibration of the aromatic amine. The narrow peaks at 2927 $cm^{-1}$ represents from C-O and the peak at 2366 $cm^{-1}$ corresponded to N-H and C-H stretching. The peak at 1600 $cm^{-1}$ corresponded to aromatic C=C bonds. The peaks at 1382 $cm^{-1}$ and 1031 $cm^{-1}$ assigned to $NO_3$ and C-N stretching of aromatic compound, respectively (Alhazmi, 2019). The peaks at 416 and 540 $cm^{-1}$ represent the AgNPs nanoparticles (Ali *et al*., 2015).

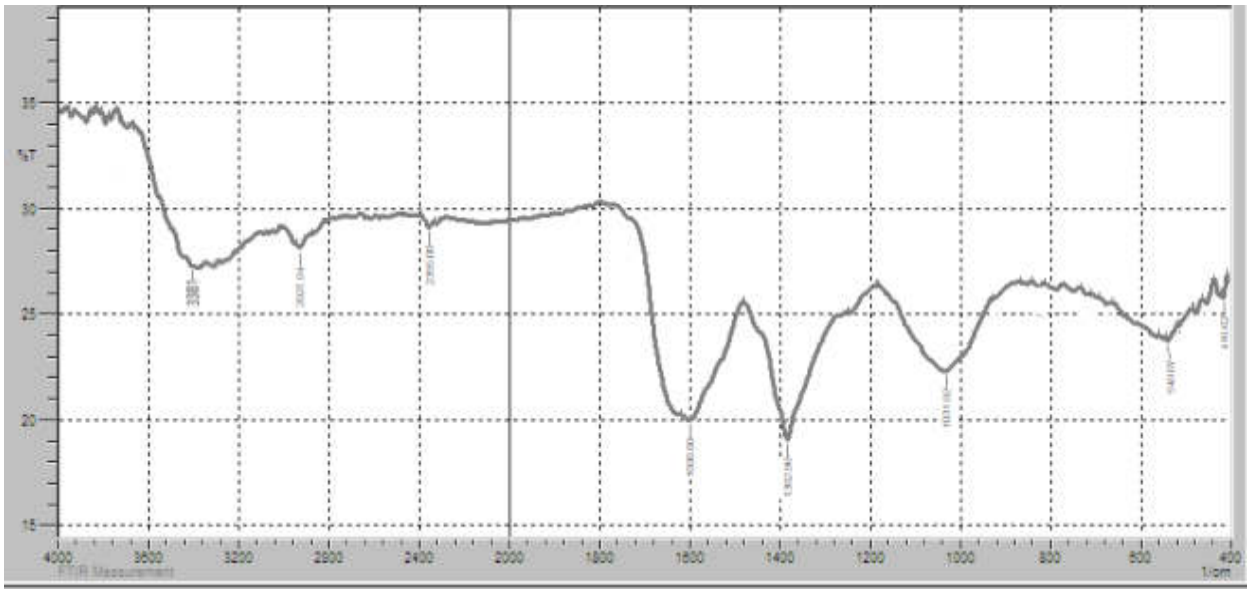


Figure (4-10) FTIR analysis of Ag/AgCl nanoparticles biosynthesized by isolates of *Lactobacillus* spp.

## 4.6 Antibacterial activity of Silver and Silver chloride Nanoparticles

### 4.6.1 Determination of antibacterial activity of Ag&AgCl nanoparticles by agar well diffusion method

Silver and silver chloride nanoparticles that biosynthesized from *Lactobacillus* spp. isolates have shown antimicrobial activity against the selected multiple drugs resist (MDR) bacteria; *P. mirabilis, E. coli, K. pneumoniae, S. aureus, S. epidermidis* and *P. aeruginosa* isolates by agar well diffusion assay. It was observed that the growth of these bacteria was inhibited at 12,500 µg/ml Ag&AgCl NPs. The nanoparticles concentrations (12,500 ; 25,000 ; 50,000 ; 100,000 µg/ml) had maximum inhibitory effect against *S. aureus* (24, 28, 30, 32mm) respectively followed by *P. mirabilis* (21, 24, 28, 30 mm) respectively, *S. epidermidis* (22, 24, 25, 28mm) respectively , *K. pneumoniae* (18, 23, 25, 28mm) respectively, *P. aeruginosa*

(19, 22, 24, 26mm) respectively and the minimum inhibitory effect against *E. coli* (16,21,23,27 mm) respectively, table (4-10) and figures (4-11), (4-12),and (4-13).

Most of the previous studies examined the effectiveness of silver nanoparticles using wells diffusion method. These studies showed weak results due to the relatively large size of the nanoparticles as shown in studies of Abbas (2018) and Mohammed (2016). The difference in the accuracy and method of dissolving the powder during preparation concentrations play an important role in the results of their effectiveness. The results of current study were in accordance with that obtained by Al-Tameme, (2017) who studied the antibacterial activity of AgNPs against *P. aeuroginosa, S. aureus, E. coli*, *K. pneumoniae* and found that the highest activity was against *S. aureus*. Another study by Kalishwaralal *et al*. (2010) who found that the anti-microbial activity of AgNPs against *P. aeruginosa* and *S. epidermidis*, was the highest at 100 µg/ml by using well-diffusion method. A study conducted by Abdel Rahim, (2015) studied the antibacterial activity of AgNPs against *S. aureus* isolates and found that the antibacterial activity of AgNPs against *S. aureus* was the highest at 100 µg/ml.

This proves the results obtained by other authors, which showed AgNPs is antibacterial agent against a wide range of microorganisms at a very low concentration. Small AgNPs with large surface areas provide an effective antimicrobial agent even at very low concentrations (Singh *et al.*, 2008). AgNPs tends to accumulate at the bacterial membrane, and form aggregates. In these conditions, several authors reported the diminution of the bacterial membrane integrity, and observed its perforations leading to the cellular death (Lemire *et al.*, 2013). If the antibacterial mechanism relied on the interaction between biological components of the cells and the AgNPs surface, so the NPs of very different sizes

from 1 nm to several hundreds of nm should not interact the same way and have the same action mechanisms (Mijnendonckx *et al*., 2013).

The antimicrobial efficacy of the nanoparticle depends on the shapes of the nanoparticles. This can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles . AgNPs with different shapes have different effects on bacterial cell. As indicated above, several studies suggest that the toxicity of AgNPs is affected by their size, which is responsible for their specific physiochemical characteristics. The smaller the nanoparticles are the larger the surface available for interaction is resulting in a higher specific activity (Sotiriou and Pratsinis, 2010). In addition, compared to larger AgNPs, small AgNPs release more Ag ions type influence the aggregation potential and toxicity of AgNPs and consequently, exhibit stronger antimicrobial activities (Xiu *et al*., 2011).

**Table (4-10) Antibacterial activity of Ag/AgCl nanoparticles by agar well Diffusion Method**.

| **Type of isolates** | **Average inhibition diameter for 12,500 µg/ml Ag&AgCl NPs concentration** | **Average inhibition diameter for 25,000 µg/ml Ag&AgCl NPs concentration** | **Average inhibition diameter for 50,000 µg/ml Ag&AgCl NPs concentration** | **Average inhibition diameter for 100,000 µg/ml Ag&AgCl NPs concentration** |
|---|---|---|---|---|
| *S. aureus* | 24 mm | 28 mm | 30 mm | 32 mm |
| *S. epidermidis* | 22 mm | 24 mm | 25 mm | 28 mm |
| *P. mirabilis* | 21 mm | 24 mm | 28 mm | 30 mm |
| *P. aeruginosa* | 19 mm | 22 mm | 24 mm | 26 mm |
| *E. coli* | 16 mm | 21 mm | 23 mm | 27 mm |
| *K. pneumoniae* | 18 mm | 23 mm | 25 mm | 28 mm |

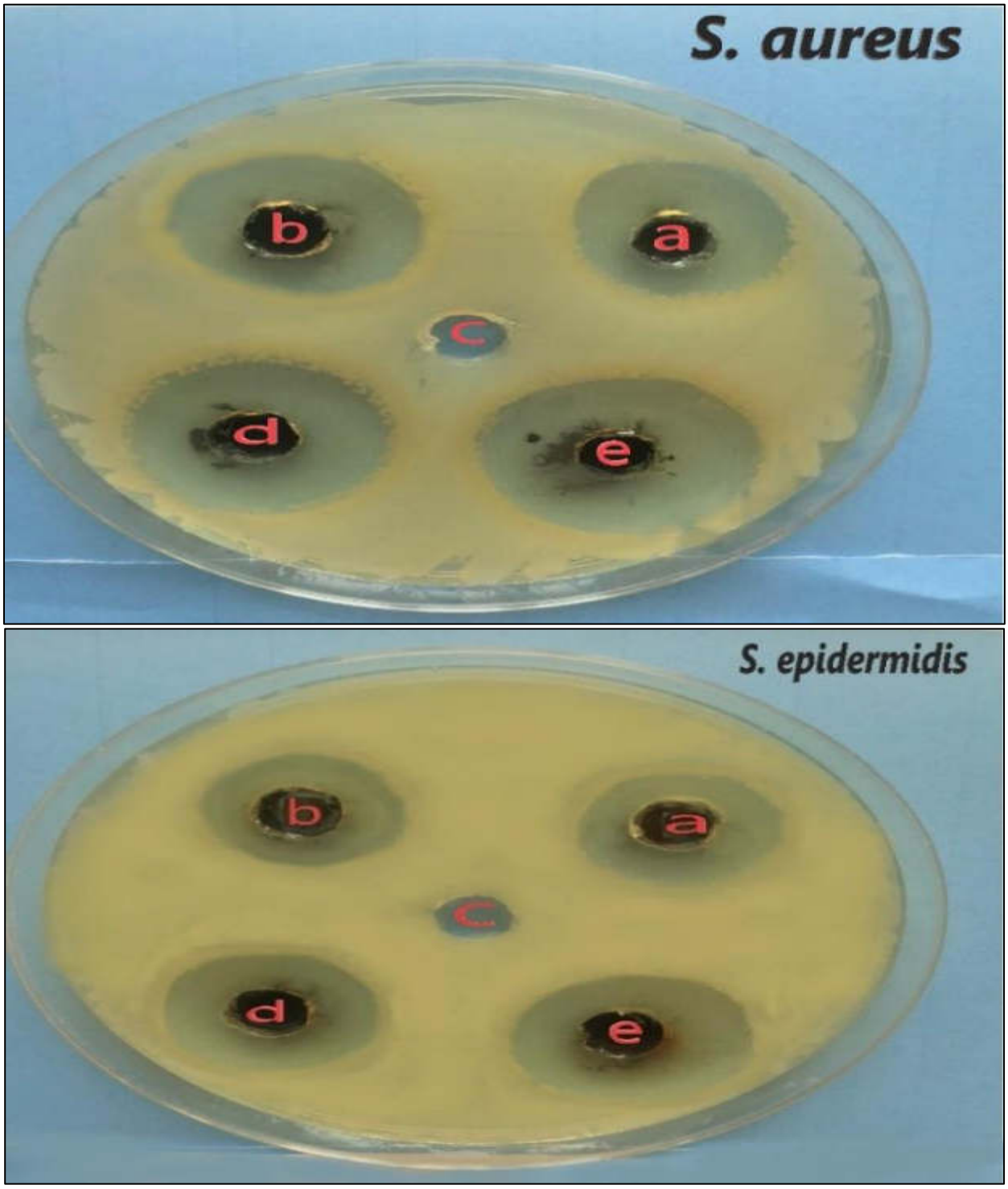



**Figure (4-11) antibacterial activity of Ag/AgCl NPs against *S. aureus* and *S. epidermidis* by agar well diffusion method. The letters (a, b, d, e,) represent the concentrations (12,500 ; 25,000; 50,000;100,000) µg/ml respectively, C (control).**

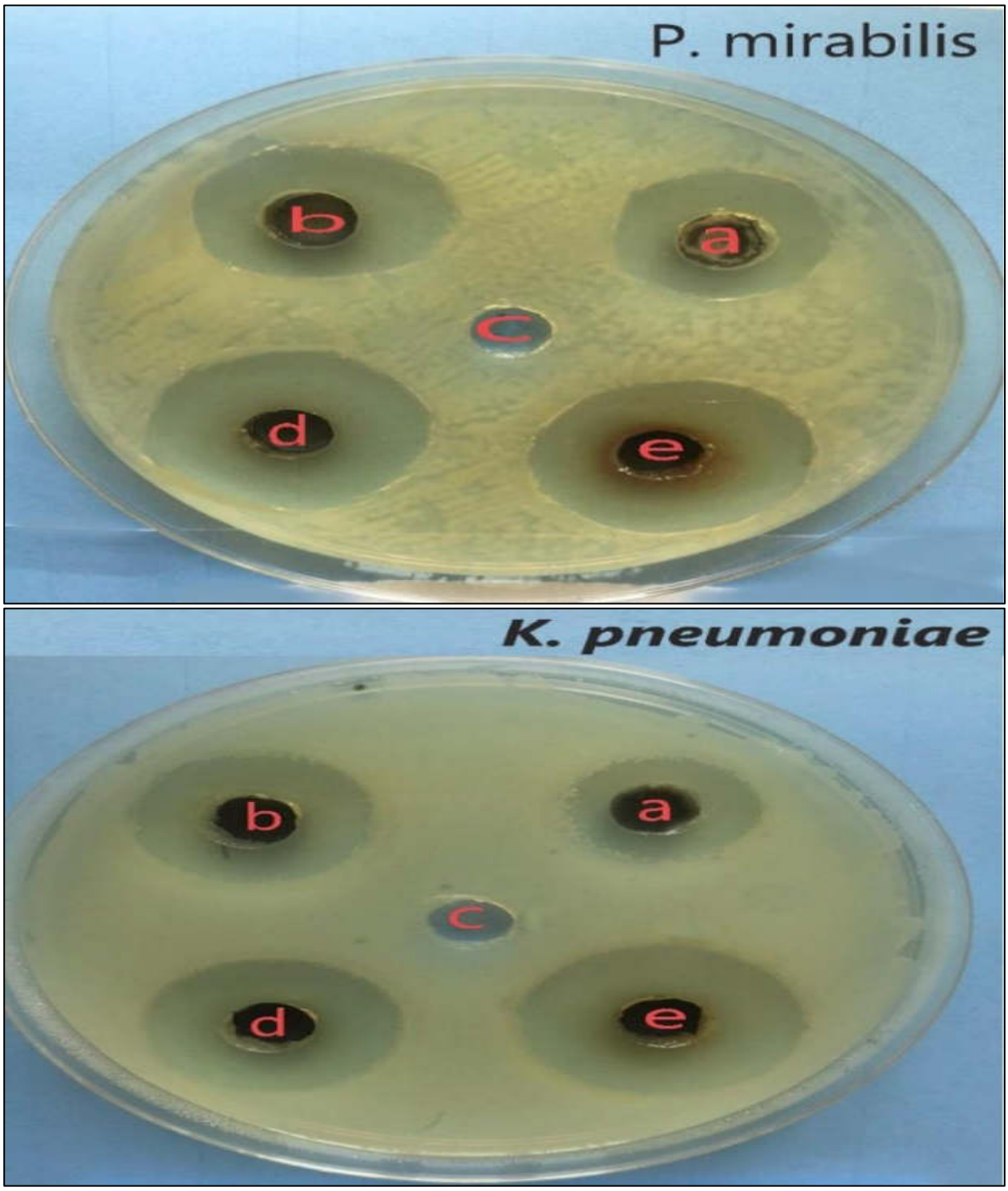



**Figure (4-12) antibacterial activity of Ag/AgCl NPs against *P. mirabilis* and *K. pneumoniae* by agar well diffusion method. The letters (a, b, d, e,) represent the concentrations (12,500 ; 25,000; 50,000;100,000) µg/ml respectively, C (control).**

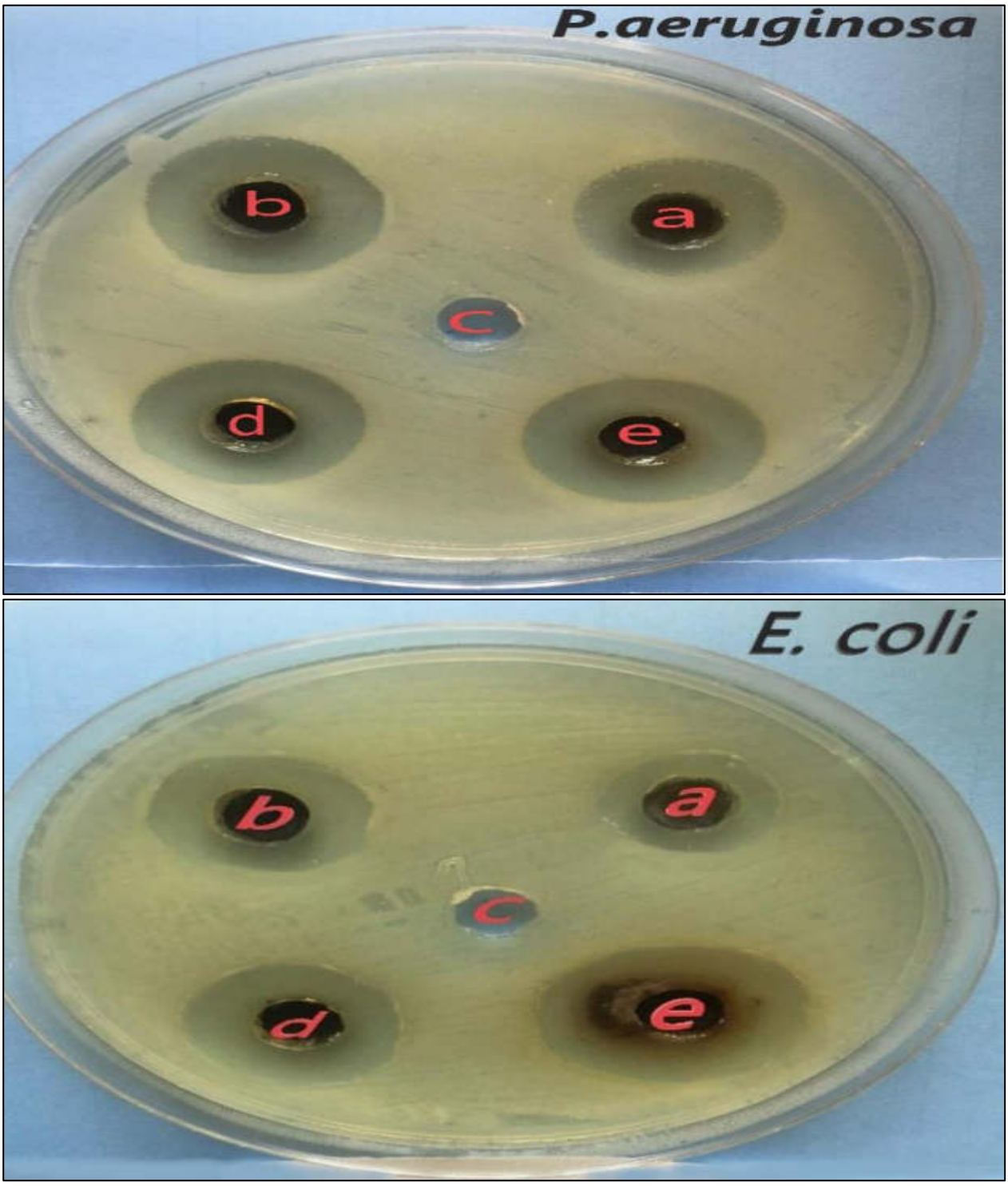



**Figure (4-13) Antibacterial activity of Ag/AgCl NPs against *P. aeruginosa* and *E. coli* by agar well diffusion method. The letters (a, b, d, e,) represent the concentrations (12,500 ; 25,000; 50,000;100,000) µg/ml respectively, C (control).**

## 4.6.2 Determination of Minimum Inhibition Concentration (MIC) of Ag/AgCl nanoparticles.

Determination of (MIC) of Ag/AgCl nanoparticles was done by microdilution method. A series of different concentrations from (2,500 - 20,000) µg/ml were performed. The result showed the MIC for Ag/AgCl NPs for *P. mirabilis, S. aureus, S. epidermidis* and *P. aeruginosa* was 2500 µg/ml while the MIC for *E. coli, K. pneumoniae* was 5000 µg/ml, table (4-11).

**Table (4-11) Minimum inhibition concentration (MIC) of Ag/AgCl nanoparticles**

| **Bacteria** | **MIC for Ag/AgCl nanoparticles** |
|---|---|
| *S. aureus* | 2500 µg/ml |
| *S. epidermidis* | 2500 µg/ml |
| *P. mirabilis* | 2500 µg/ml |
| *P. aeruginosa* | 2500 µg/ml |
| *E. coli* | 5000 µg/ml |
| *K. pneumoniae* | 5000 µg/ml |

# Conclusions and Recommendations

# Conclusions

1. *K. pneumoniae* was the most common isolated bacteria from burn, wound and urine, comparative with *S. aureus* and *E. coli*. While *P. aeruginosa*, *S. epidermidis* and *P. mirabilis* which were found in a low percentage of occurrence.
2. *K. pneumoniae* possess the highest rate of multiple drugs resistant followed by *E. coli, S. aureus, P. aeruginosa, P. mirabilis* and *S. epidermidis*.
3. The appearance of resistant isolates of *S. aureus* and *S. epidermidis* bacteria to Vancomycin antibiotic gives a dangerous indication of overuse and abuse of antibiotics. In particular, vancomycin is considered the last choose for the treatment of methicillin-resistant *S. aureus* and *S. epidermidis* infections.
4. The biosynthesis of AgNPs mediated by the filtrated CFS(whey) of *Lactobacillus* spp. Which isolated and identified from different types of local dairy products, after grown in skim milk, considered a cheap and good way to produce nanoparticles with small size.
5. The biosynthesized silver nanoparticles were Characterized using X-ray diffraction (XRD) analysis confirms the nanoparticles are silver and silver chloride (AgCl) cubic types. The (crystalline) domains equation, showed three strongest peaks; the calculated particle size were 17.1and 18.6 nm for Ag, and 24.3 nm for AgCl.
6. Atomic Force Microscopy (AFM) used to characterize the size, topography and granularity volume distribution of biosynthesized nanoparticles Ag/AgCl, average size was 50 nm.

7. The antibacterial activity of silver and sliver chloride nanoparticles against the selected multiple drugs resist (MDR) bacteria by agar well diffusion method appeared that the growth of these bacteria was inhibited and the maximum inhibitory effect against *S. aureus* and minimum inhibitory effect against *E. coli* while the others bacteria (*P. mirabilis, S. epidermidis, K. pneumoniae and P. aeruginosa*) inhibitory effect varied between the bacterial isolates and the concentration of the silver nanoparticles

8. The biosynthesized Ag/AgCl nanoparticles have the most strong antibacterial activity against *S. aureus* followed by *P. mirabilis, S. epidermidis, K. pneumoniae, P. aeruginosa and* then *E. coli*.

9. The biosynthesized Ag/AgCl NPs were more effective than the most common antibiotic.

# Recommendation

1- Comparison the efficiency of biosynthesized silver nanoparticles from other different bacteria.

2- Comparison the efficiency of the biosynthesized silver nanoparticles with Ag NPs produced by other methods (physical and chemical methods)against the tested pathogens.

3- Studying the activity of biosynthesized silver nanoparticles in treatment of drinking water.

4- Studying the antifungal activity of biosynthesized silver nanoparticles.

5- Designing an experimental model for determination of the biological parameter in *vivo* study.

6- Study the effect of environmental factor like temperature, pH, the concentration of the substrate in the production of Sliver nanoparticles

7- Studying the effect of the thermal treatment on the size and shape of produced nanoparticles.

8- Study Ag/AgCl nanoparticles effect on bacteria after exposure to them.

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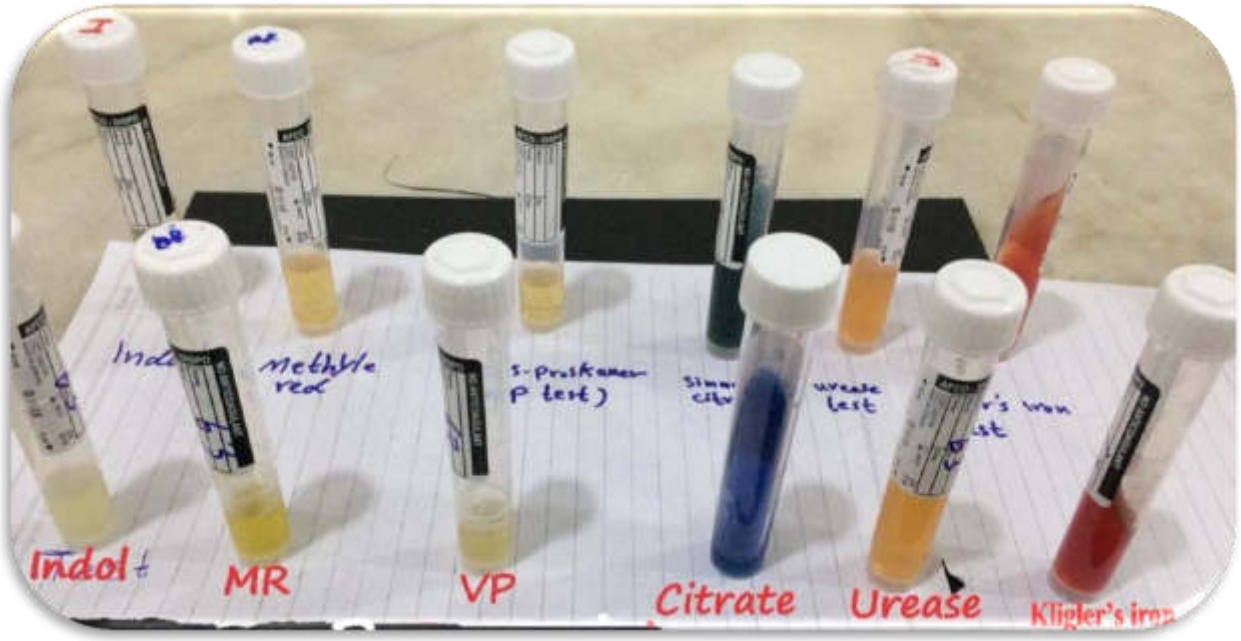
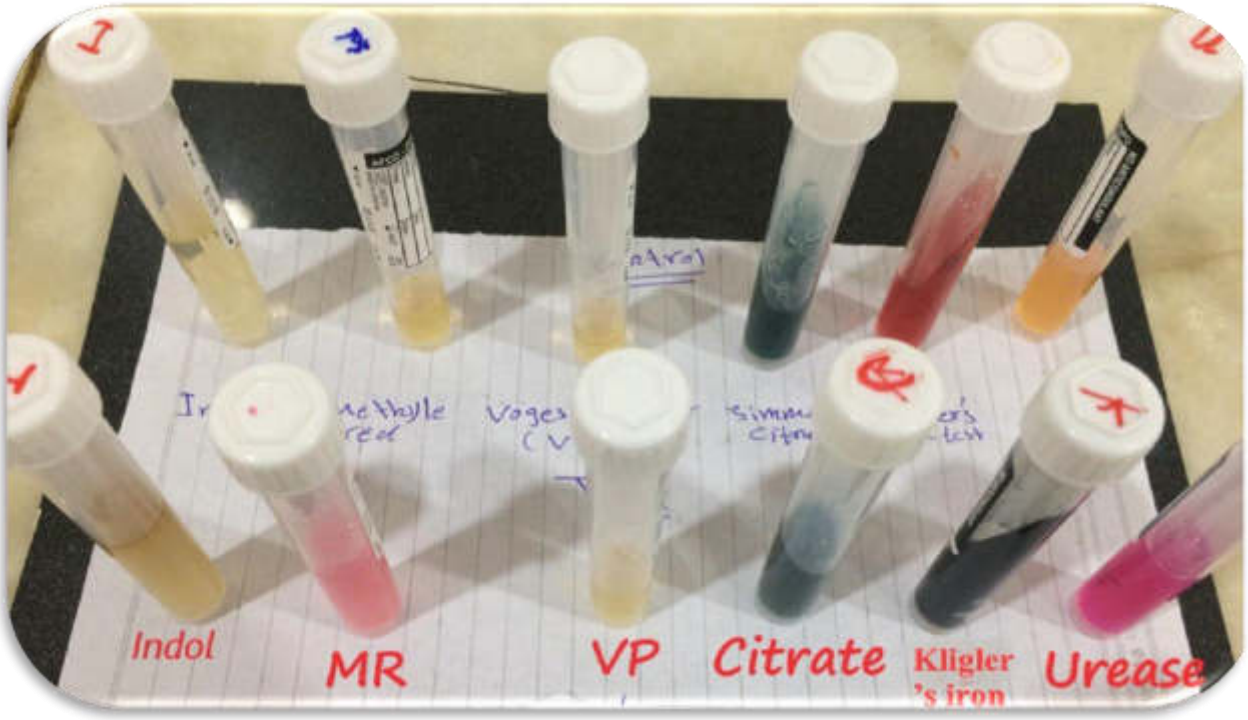
# Appendix

# Appendix




Figure(1-2) *P. aeruginosa* IMViC test result



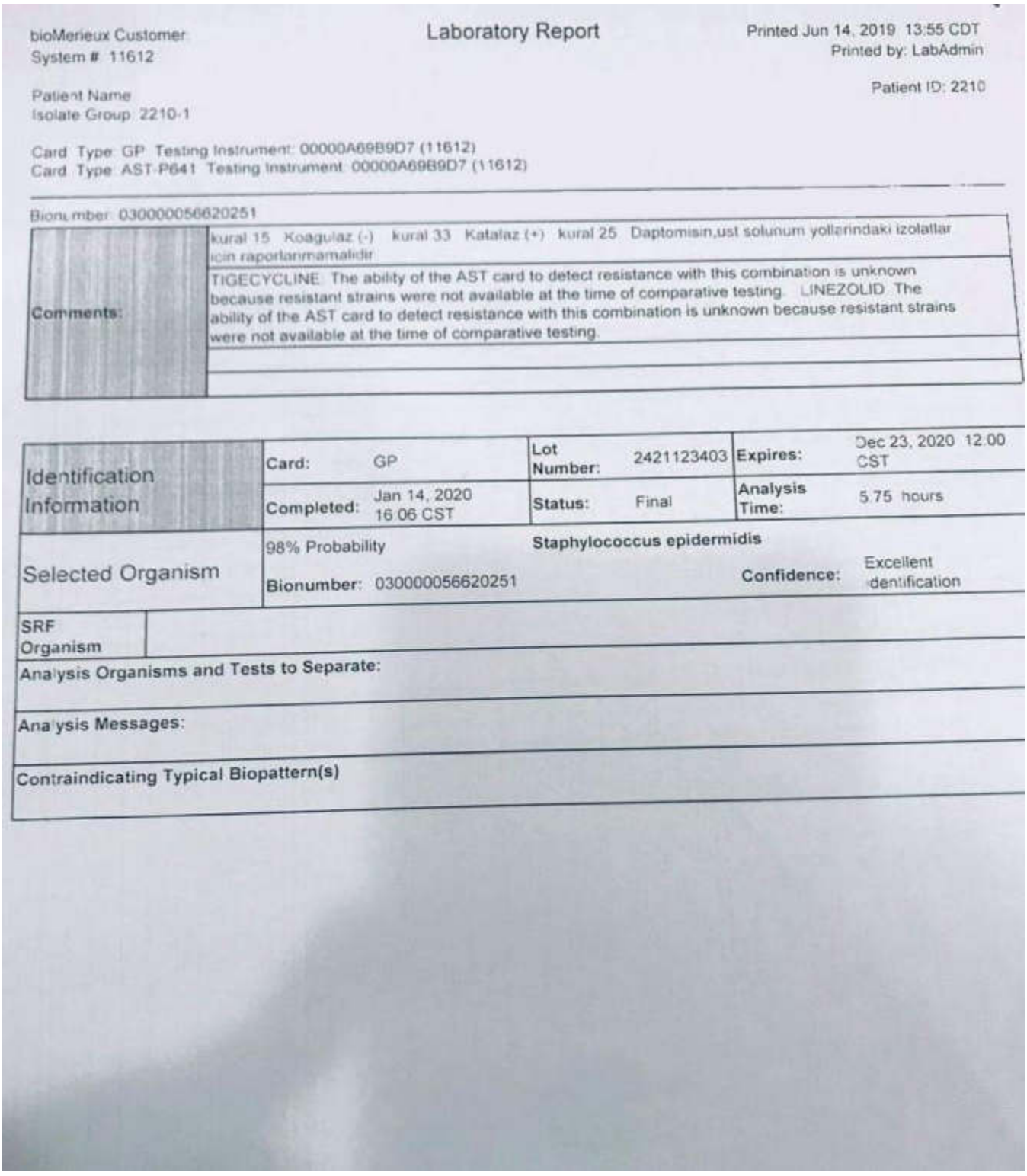

Figure(1-2) *P. mirabilis* IMViC test result

# Appendix


Figure(1-3) *E. coli* IMViC test result


Figure(1-4) *K. pneumonia* IMViC test result

# Appendix



bioMerieux Customer
System # 11612

**Laboratory Report**

Printed Jun 14, 2019 13:55 CDT
Printed by: LabAdmin

Patient ID: 2210

Patient Name
Isolate Group: 2210-1

Card Type: GP Testing Instrument: 00000A69B9D7 (11612)
Card Type: AST-P641 Testing Instrument: 00000A69B9D7 (11612)

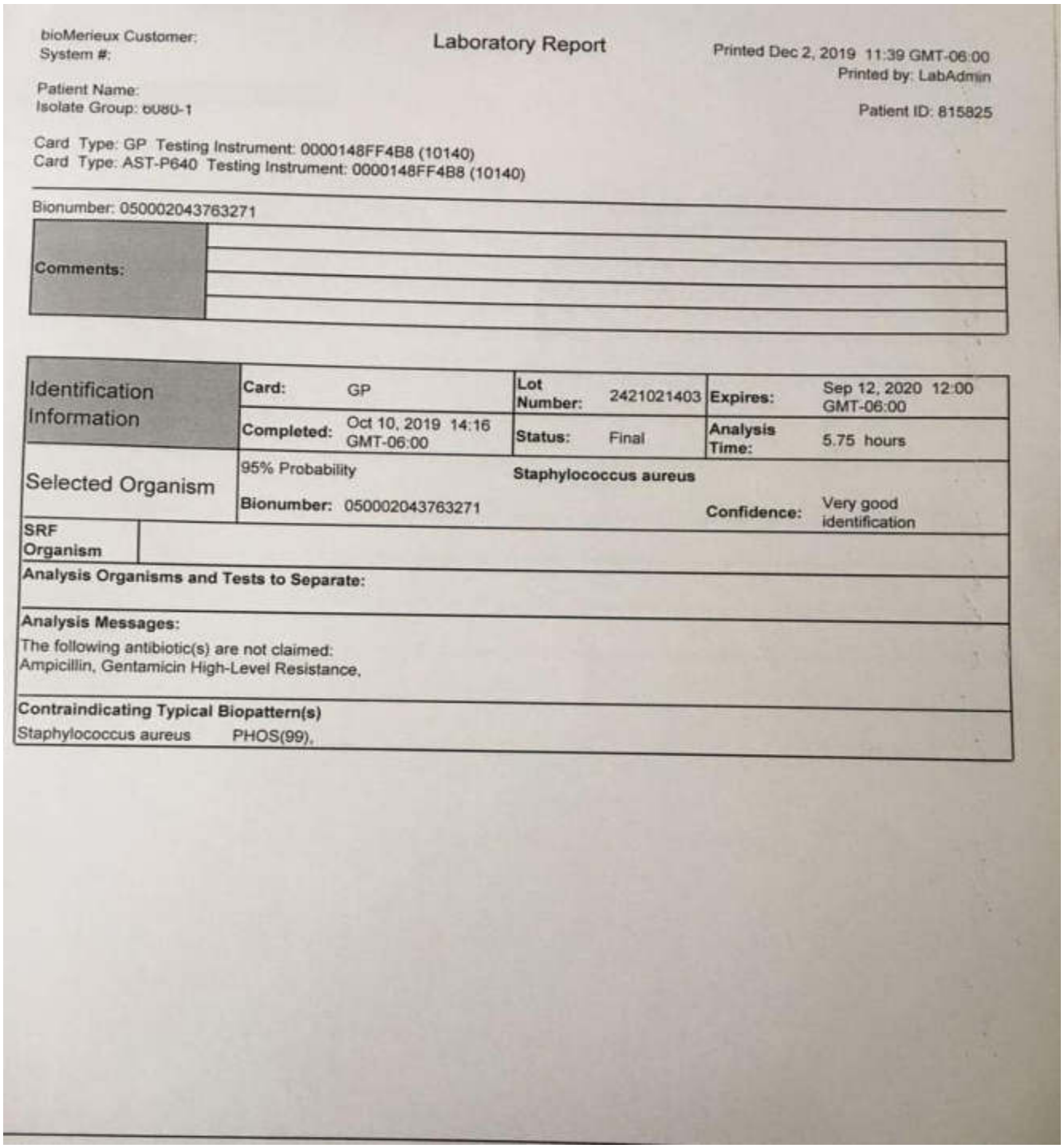
Bionumber: 030000056620251

| | |
|---|---|
| **Comments:** | kural 15 Koagulaz (-) kural 33 Katalaz (+) kural 25 Daptomisin,ust solunum yollarindaki izolatlar icin raporlanmamalidir |
| | TIGECYCLINE: The ability of the AST card to detect resistance with this combination is unknown because resistant strains were not available at the time of comparative testing. LINEZOLID: The ability of the AST card to detect resistance with this combination is unknown because resistant strains were not available at the time of comparative testing. |
| | |
| | |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | **Card:** | GP | **Lot Number:** | 2421123403 | **Expires:** Dec 23, 2020 12:00 CST |
| | **Completed:** | Jan 14, 2020 16:06 CST | **Status:** | Final | **Analysis Time:** 5.75 hours |
| Selected Organism | 98% Probability | | **Staphylococcus epidermidis** | | |
| | **Bionumber:** | 030000056620251 | | | **Confidence:** Excellent identification |
| **SRF Organism** | | | | | |
| **Analysis Organisms and Tests to Separate:** | | | | | |
| **Analysis Messages:** | | | | | |
| **Contraindicating Typical Biopattern(s)** | | | | | |

Figure(1-5) Confirm identification of *Staphylococcus epidermidis* isolate by VITEK 2 system

# Appendix



bioMerieux Customer:
System #:

**Laboratory Report**

Printed Dec 2, 2019 11:39 GMT-06:00
Printed by: LabAdmin

Patient Name:
Isolate Group: 6080-1

Patient ID: 815825

Card Type: GP Testing Instrument: 0000148FF4B8 (10140)
Card Type: AST-P640 Testing Instrument: 0000148FF4B8 (10140)

Bionumber: 050002043763271

| | |
|---|---|
| **Comments:** | |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | **Card:** | GP | **Lot Number:** | 2421021403 | **Expires:** Sep 12, 2020 12:00 GMT-06:00 |
| | **Completed:** | Oct 10, 2019 14:16 GMT-06:00 | **Status:** | Final | **Analysis Time:** 5.75 hours |
| Selected Organism | 95% Probability | | **Staphylococcus aureus** | | |
| | **Bionumber:** | 050002043763271 | | | **Confidence:** Very good identification |
| **SRF Organism** | | | | | |

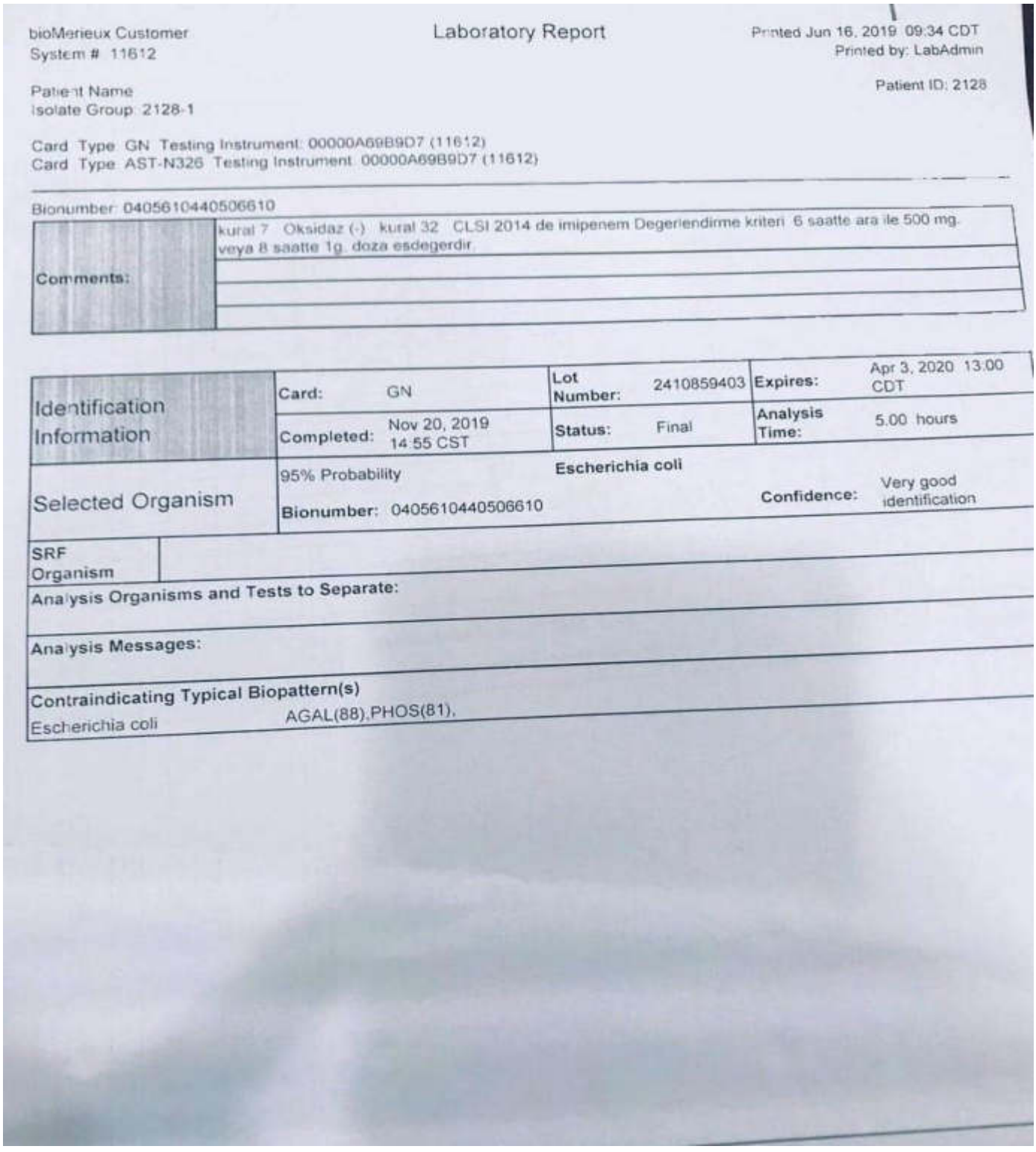
**Analysis Organisms and Tests to Separate:**

**Analysis Messages:**
The following antibiotic(s) are not claimed:
Ampicillin, Gentamicin High-Level Resistance,

**Contraindicating Typical Biopattern(s)**
Staphylococcus aureus PHOS(99),

Figure(1-6) Confirm identification of *Staphylococcus aureus* isolate by VITEK 2 system

# Appendix



bioMerieux Customer
System # 11612

Laboratory Report

Printed Jun 16, 2019 09:34 CDT
Printed by: LabAdmin

Patient Name
Isolate Group: 2128-1

Patient ID: 2128

Card Type: GN Testing Instrument: 00000A69B9D7 (11612)
Card Type: AST-N326 Testing Instrument: 00000A69B9D7 (11612)

Bionumber: 0405610440506610

| | |
|---|---|
| Comments: | kural 7 Oksidaz (-) kural 32 CLSI 2014 de imipenem Degeriendirme kriteri 6 saatte ara ile 500 mg. veya 8 saatte 1g. doza esdegerdir. |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | Card: | GN | Lot Number: | 2410859403 | Expires: Apr 3, 2020 13:00 CDT |
| | Completed: | Nov 20, 2019 14:55 CST | Status: | Final | Analysis Time: 5.00 hours |
| Selected Organism | 95% Probability | | Escherichia coli | | Confidence: Very good identification |
| | Bionumber: | 0405610440506610 | | | |
| SRF Organism | | | | | |

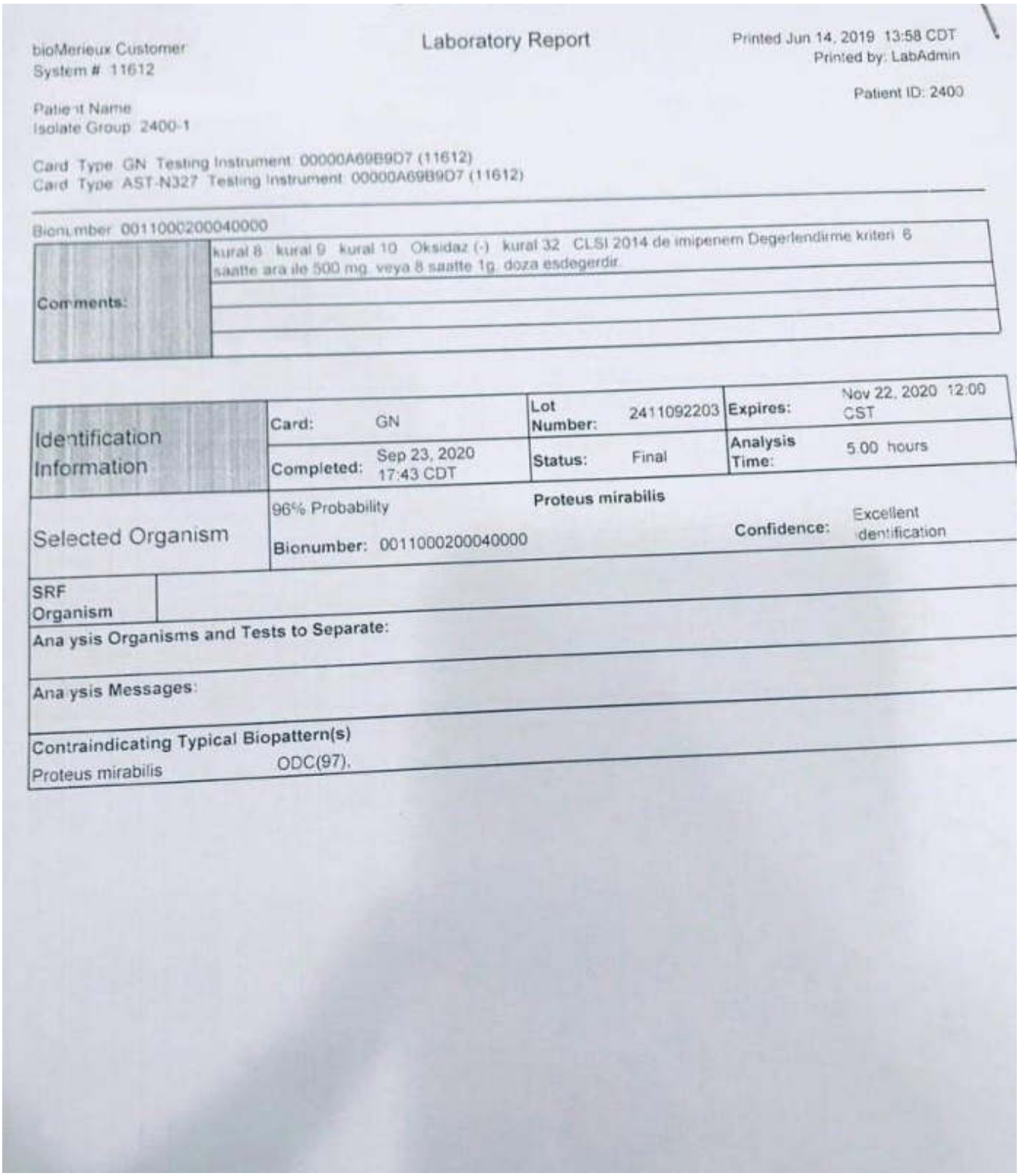
Analysis Organisms and Tests to Separate:

Analysis Messages:

Contraindicating Typical Biopattern(s)
Escherichia coli AGAL(88),PHOS(81),

Figure(1-7) Confirm identification of *Escherichia coli* isolate by VITEK 2 system

# Appendix



bioMerieux Customer
System # 11612

Laboratory Report

Printed Jun 14, 2019 13:58 CDT
Printed by: LabAdmin

Patient ID: 2400

Patient Name
Isolate Group 2400-1

Card Type GN Testing Instrument 00000A69B9D7 (11612)
Card Type AST-N327 Testing Instrument 00000A69B9D7 (11612)

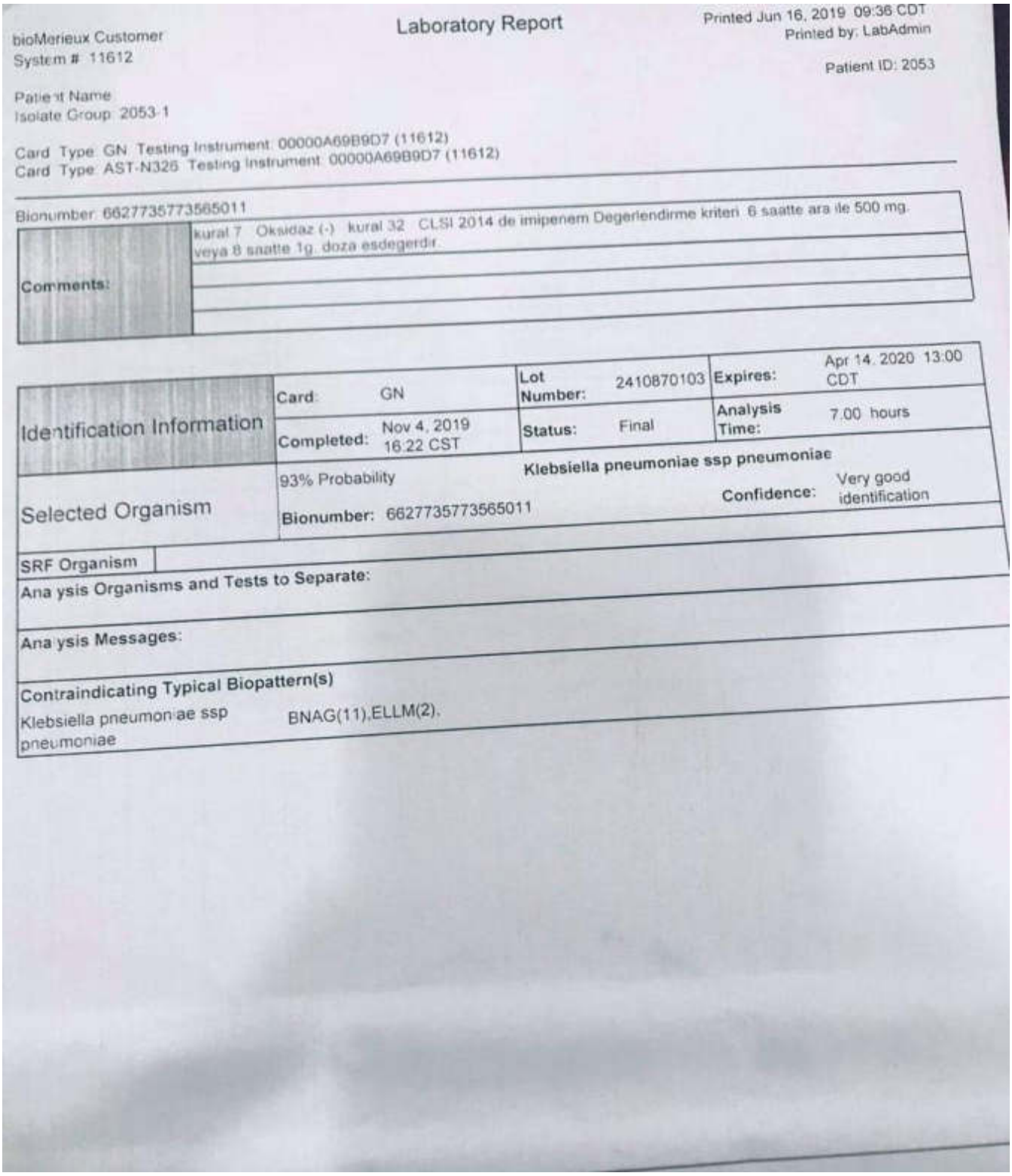
Bionumber 0011000200040000

| | |
|---|---|
| Comments: | kural 8 kural 9 kural 10 Oksidaz (-) kural 32 CLSI 2014 de imipenem Degerlendirme kriteri 6 saatte ara ile 500 mg, veya 8 saatte 1g, doza esdegerdir. |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | Card: | GN | Lot Number: | 2411092203 | Expires: Nov 22, 2020 12:00 CST |
| | Completed: | Sep 23, 2020 17:43 CDT | Status: | Final | Analysis Time: 5.00 hours |
| Selected Organism | 96% Probability | | Proteus mirabilis | | Confidence: Excellent identification |
| | Bionumber: 0011000200040000 | | | | |
| SRF Organism | | | | | |
| Ana ysis Organisms and Tests to Separate: | | | | | |
| Ana ysis Messages: | | | | | |
| Contraindicating Typical Biopattern(s) Proteus mirabilis ODC(97). | | | | | |

Figure(1-8) Confirm identification of *Proteus mirabilis* isolate by VITEK 2 system

# Appendix



Laboratory Report

bioMerieux Customer
System # 11612

Printed Jun 16, 2019 09:36 CDT
Printed by: LabAdmin

Patient ID: 2053

Patient Name
Isolate Group: 2053-1

Card Type: GN Testing Instrument: 00000A69B9D7 (11612)
Card Type: AST-N326 Testing Instrument: 00000A69B9D7 (11612)

Bionumber: 6627735773565011

| | |
|---|---|
| Comments: | kural 7 Oksidaz (-) kural 32 CLSI 2014 de imipenem Degerlendirme kriteri 6 saatte ara ile 500 mg. veya 8 saatte 1g. doza esdegerdir. |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | Card: | GN | Lot Number: | 2410870103 | Expires: Apr 14, 2020 13:00 CDT |
| | Completed: | Nov 4, 2019 16:22 CST | Status: | Final | Analysis Time: 7.00 hours |
| Selected Organism | 93% Probability | | Klebsiella pneumoniae ssp pneumoniae | | Confidence: Very good identification |
| | Bionumber: 6627735773565011 | | | | |
| SRF Organism | | | | | |

Ana ysis Organisms and Tests to Separate:

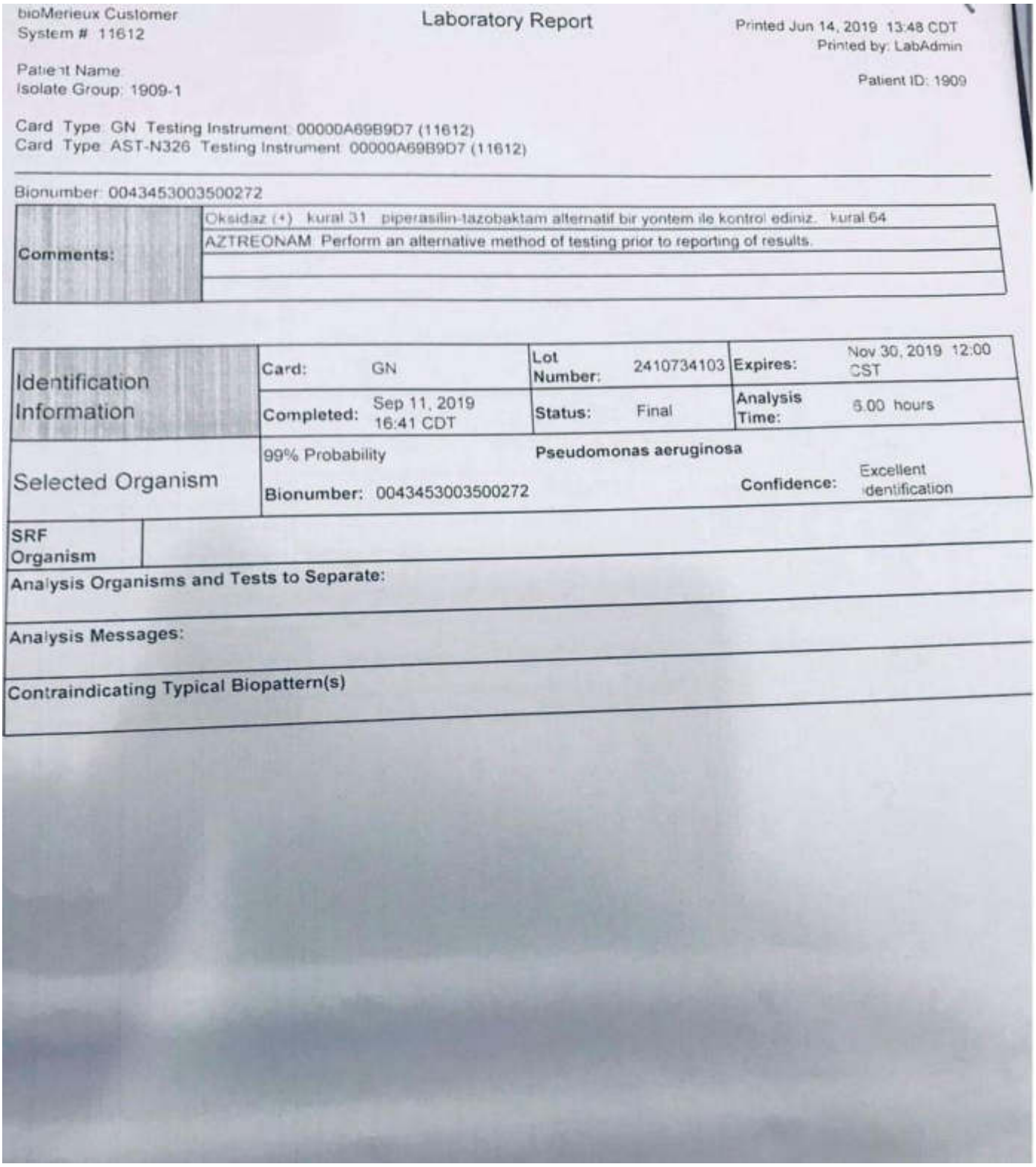
Ana ysis Messages:

Contraindicating Typical Biopattern(s)

| | |
|---|---|
| Klebsiella pneumon ae ssp pneumoniae | BNAG(11),ELLM(2). |

Figure(1-9) Confirm identification of *Klebsiella pneumoniae* isolate by VITEK 2 system

# Appendix



bioMerieux Customer
System # 11612

Laboratory Report

Printed Jun 14, 2019 13:48 CDT
Printed by: LabAdmin

Patient Name
Isolate Group: 1909-1

Patient ID: 1909

Card Type: GN Testing Instrument: 00000A69B9D7 (11612)
Card Type: AST-N326 Testing Instrument: 00000A69B9D7 (11612)

Bionumber: 0043453003500272

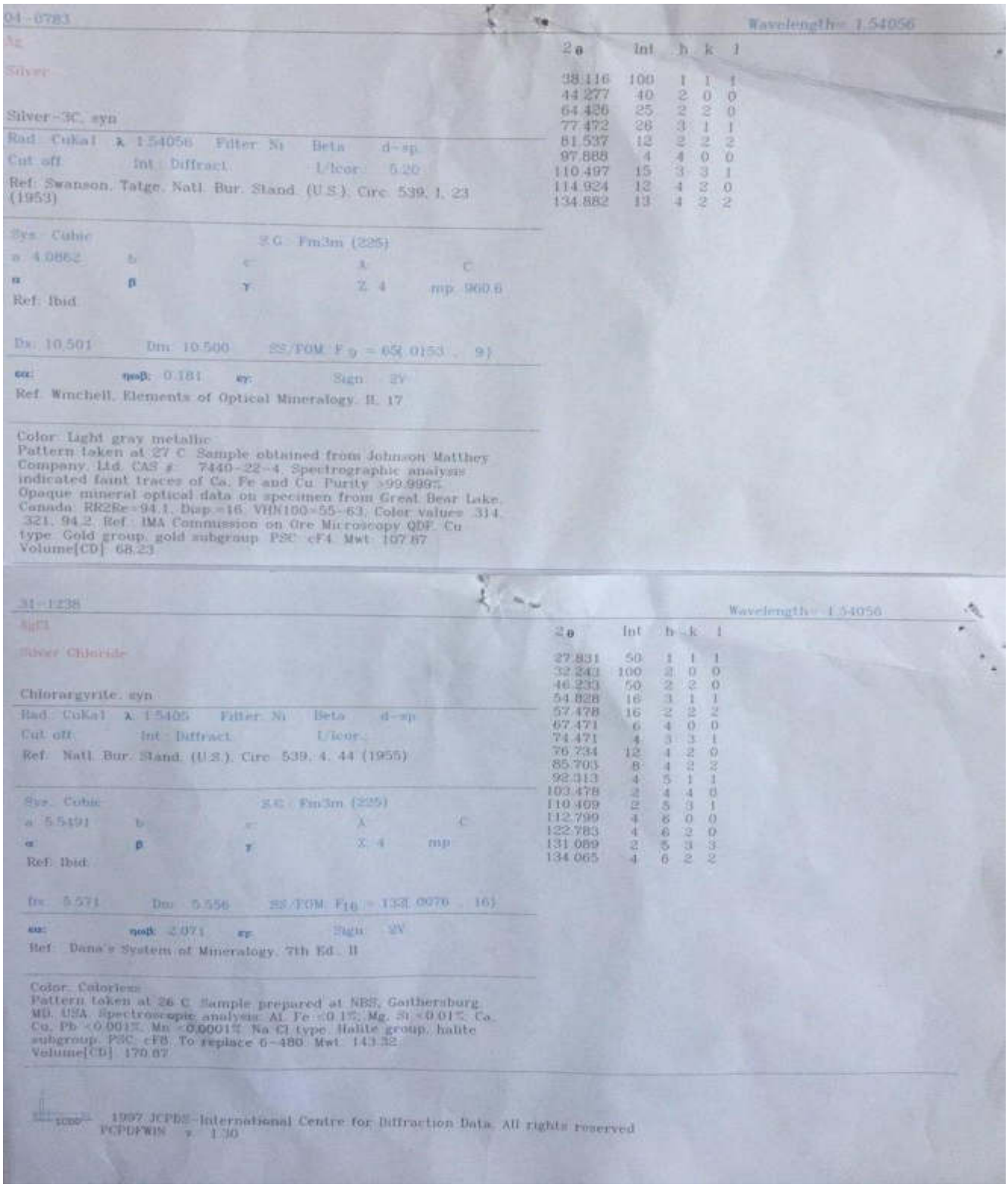
| | |
|---|---|
| Comments: | Oksidaz (+) kural 31 piperasilin-tazobaktam alternatif bir yontem ile kontrol ediniz. kural 64 |
| | AZTREONAM: Perform an alternative method of testing prior to reporting of results. |
| | |
| | |

| | | | | | |
|---|---|---|---|---|---|
| Identification Information | Card: | GN | Lot Number: | 2410734103 | Expires: | Nov 30, 2019 12:00 CST |
| | Completed: | Sep 11, 2019 16:41 CDT | Status: | Final | Analysis Time: | 6.00 hours |
| Selected Organism | 99% Probability | | Pseudomonas aeruginosa | | | |
| | Bionumber: | 0043453003500272 | | | Confidence: | Excellent identification |

| | |
|---|---|
| SRF Organism | |
| Analysis Organisms and Tests to Separate: | |
| Analysis Messages: | |
| Contraindicating Typical Biopattern(s) | |

Figure(1-10) Confirm identification of *Pseudomonas aeruginosa* isolate by VITEK 2 system

# Appendix



04-0783

Ag

Silver

Silver-3C, syn

Rad. CuKa1 λ 1.54056 Filter: Ni Beta d-sp

Cut off Int: Diffract. I/Icor. 5.20

Ref: Swanson, Tatge, Natl. Bur. Stand. (U.S.), Circ. 539, 1, 23 (1953)

Sys: Cubic S.G.: Fm3m (225)

a: 4.0862 b: c: A: C:

α β γ Z: 4 mp: 960.8

Ref: Ibid.

Dx: 10.501 Dm: 10.500 SS/FOM: F 9 = 65( 0.153 , 9)

εα: ηαβ: 0.181 εγ: Sign: 2V

Ref: Winchell, Elements of Optical Mineralogy, II, 17

Color: Light gray metallic

Pattern taken at 27 C. Sample obtained from Johnson Matthey Company, Ltd. CAS #: 7440-22-4. Spectrographic analysis indicated faint traces of Ca, Fe and Cu. Purity >99.999%. Opaque mineral optical data on specimen from Great Bear Lake, Canada. RR2Re=94.1, Disp.=16. VHN100=55-63. Color values: 314, 321, 94.2. Ref: IMA Commission on Ore Microscopy QDF. Cu type. Gold group, gold subgroup. P2C. cF4. Mwt: 107.87. Volume[CD]: 68.23

| 2θ | Int | h | k | l |
|---|---|---|---|---|
| 38.116 | 100 | 1 | 1 | 1 |
| 44.277 | 40 | 2 | 0 | 0 |
| 64.426 | 25 | 2 | 2 | 0 |
| 77.472 | 26 | 3 | 1 | 1 |
| 81.537 | 12 | 2 | 2 | 2 |
| 97.888 | 4 | 4 | 0 | 0 |
| 110.497 | 15 | 3 | 3 | 1 |
| 114.924 | 12 | 4 | 2 | 0 |
| 134.882 | 13 | 4 | 2 | 2 |

Wavelength= 1.54056

31-1238

AgCl

Silver Chloride

Chlorargyrite, syn

Rad. CuKa1 λ 1.5405 Filter: Ni Beta d-sp

Cut off: Int: Diffract. I/Icor.:

Ref: Natl. Bur. Stand. (U.S.), Circ. 539, 4, 44 (1955)

Sys: Cubic S.G.: Fm3m (225)

a: 5.5491 b: c: A: C:

α β γ Z: 4 mp:

Ref: Ibid.

Dx: 5.571 Dm: 5.556 SS/FOM: F16 = 133.0070 , 16)

εα: ηαβ: 2.071 εγ: Sign: 2V

Ref: Dana's System of Mineralogy, 7th Ed., II

Color: Colorless

Pattern taken at 26 C. Sample prepared at NBS, Gaithersburg, MD, USA. Spectroscopic analysis: Al, Fe <0.1%; Mg, Si <0.01%; Ca, Cu, Pb <0.001%; Mn <0.0001%. Na Cl type. Halite group, halite subgroup. P3C. cF8. To replace 6-480. Mwt: 143.32. Volume[CD]: 170.87

| 2θ | Int | h | k | l |
|---|---|---|---|---|
| 27.831 | 50 | 1 | 1 | 1 |
| 32.243 | 100 | 2 | 0 | 0 |
| 46.230 | 50 | 2 | 2 | 0 |
| 54.828 | 16 | 3 | 1 | 1 |
| 57.478 | 16 | 2 | 2 | 2 |
| 67.471 | 6 | 4 | 0 | 0 |
| 74.471 | 4 | 3 | 3 | 1 |
| 76.734 | 12 | 4 | 2 | 0 |
| 85.703 | 8 | 4 | 2 | 2 |
| 92.313 | 4 | 5 | 1 | 1 |
| 103.478 | 2 | 4 | 4 | 0 |
| 110.409 | 2 | 5 | 3 | 1 |
| 112.799 | 4 | 6 | 0 | 0 |
| 122.783 | 4 | 6 | 2 | 0 |
| 131.089 | 2 | 5 | 3 | 3 |
| 134.065 | 4 | 6 | 2 | 2 |

Wavelength= 1.54056



Figure(1-11) X-ray Diffraction (XRD) analysis diagnoses

# Appendix

**Table (1-1) *E. coli* sensitivity test and sources**

| Antibiotics | *E. coli* inhibitory zone(mm) | Zone Diameter (mm) Interpretive Criteria (CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Imipenem** | R/17 mm | ≥23 | ≤19 | 20-22 |
| **Tetracycline** | S/20 mm | ≥15 | ≤11 | 12-14 |
| **Doxycycline** | S/25 mm | ≥14 | ≤10 | 11-13 |
| **Aztreonam** | R/0 mm | ≥21 | ≤17 | 18-20 |
| **Levofloxacin** | I/20 mm | ≥21 | ≤16 | 17-20 |
| **Cefpodoxime** | R/0 mm | ≥21 | ≤17 | 18-20 |
| **Trimethoprim-sulfamethoxazole** | R/0 mm | ≥16 | ≤10 | 11-15 |
| **Azithromycin** | R/0 mm | ≥13 | ≤12 | - |
| **Ampicillin-sulbactam** | R/0 mm | ≥15 | ≤11 | 12-14 |
| **Ticarcillin-clavulanate** | R/8 mm | ≥20 | ≤14 | 15-19 |
| **Cefuroxime** | R/0 mm | ≥18 | ≤14 | 15-17 |
| **Cefoxitin** | R/0 mm | ≥18 | ≤14 | 15-17 |
| **Source** | Urinary tract infection | | | |

# Appendix

**Table(1-2) *K. pneumoniae* sensitivity test and sources**

| Antibiotics | *K. pneumoniae* inhibitory zone (mm) | Zone Diameter (mm) Interpretive Criteria(CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Ciprofloxacin** | R/0 mm | ≥26 | ≤21 | 22-25 |
| **Ofloxacin** | R/0 mm | ≥16 | ≤12 | 13-15 |
| **Gentamicin** | R/0 mm | ≥15 | ≤12 | 13-14 |
| **Trimethoprim** | R/0 mm | ≥16 | ≤10 | 11-15 |
| **Cefotaxime** | R/0 mm | ≥26 | ≤22 | 23-25 |
| **Cefuroxime** | R/0 mm | ≥18 | ≤14 | 15-17 |
| **Amikacin** | R/12 mm | ≥17 | ≤14 | 15-16 |
| **Streptomycin** | R/7 mm | ≥15 | ≤11 | 12-14 |
| **Nitrofurantoin** | R/0 mm | ≥17 | ≤14 | 15-16 |
| **Piperacillin** | R/0 mm | ≥21 | ≤17 | 18-20 |
| **Imipenem** | R/10 mm | ≥23 | ≤19 | 20-22 |
| **Chloramphenicol** | R/0 mm | ≥18 | ≤12 | 13-17 |
| **Source** | Burn infection | | | |

# Appendix

**Table (1-3) *P. mirabilis* sensitivity test and source**

| Antibiotics | *P. mirabilis* inhibitory zone (mm) | Zone Diameter (mm) Interpretive Criteria(CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Ciprofloxacin** | S/25 mm | ≥26 | ≤21 | 22-25 |
| **Ofloxacin** | S/23 mm | ≥16 | ≤12 | 13-15 |
| **Gentamicin** | R/0 mm | ≥15 | ≤12 | 13-14 |
| **Trimethoprim** | R/0 mm | ≥16 | ≤10 | 11-15 |
| **Cefotaxime** | R/17mm | ≥26 | ≤22 | 23-25 |
| **Cefuroxime** | R/8 mm | ≥18 | ≤14 | 15-17 |
| **Amikacin** | R/10 mm | ≥17 | ≤14 | 15-16 |
| **Streptomycin** | R/0 mm | ≥15 | ≤11 | 12-14 |
| **Nitrofurantoin** | R/13 mm | ≥17 | ≤14 | 15-16 |
| **Piperacillin** | S/21 mm | ≥21 | ≤17 | 18-20 |
| **Imipenem** | S/23 mm | ≥23 | ≤19 | 20-22 |
| **Chloramphenicol** | R/9 mm | ≥18 | ≤12 | 13-17 |
| **Source** | wound infection | | | |

# Appendix

**Table(1-4) *P. aeruginosa* sensitivity test and source**

| Antibiotics | *P. aeruginosa* inhibitory zone (mm) | Zone Diameter (mm) Interpretive Criteria(CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Ciprofloxacin** | S/30 mm | ≥25 | ≤18 | 19-24 |
| **Ofloxacin** | S/21 mm | ≥16 | ≤12 | 13-15 |
| **Gentamicin** | R/12 mm | ≥15 | ≤12 | 13-14 |
| **Tobramycin** | R/7 mm | ≥15 | ≤12 | 13-14 |
| **Aztreonam** | R/0 mm | ≥22 | ≤15 | 16-21 |
| **Levofloxacin** | S/23 mm | ≥22 | ≤14 | 15-21 |
| **Amikacin** | R/14 mm | ≥17 | ≤14 | 15-16 |
| **Meropenem** | R/0 mm | ≥19 | ≤15 | 16-18 |
| **Ceftazidime** | R/0 mm | ≥18 | ≤14 | 15-17 |
| **Piperacillin** | R/9 mm | ≥21 | ≤14 | 15-20 |
| **Imipenem** | R/5 | ≥19 | ≤15 | 16-18 |
| **Cefepime** | R/12 | ≥18 | ≤14 | 15-17 |
| **Source** | Burn infection | | | |

# Appendix

**Table (1-5) *S. aureus* sensitivity test and source**

| Antibiotics | *S. aureus* inhibitory zone (mm) | Zone Diameter (mm) Interpretive Criteria(CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Ciprofloxacin** | R/14 mm | ≥21 | ≤15 | 16-20 |
| **Ofloxacin** | R/13 mm | ≥18 | ≤14 | 15-17 |
| **Levofloxacin** | R/13 mm | ≥19 | ≤15 | 16-18 |
| **Azithromycin** | R/0 mm | ≥18 | ≤13 | 14-17 |
| **Clarithromycin** | R/0 mm | ≥18 | ≤13 | 14-17 |
| **Tetracycline** | R/9 mm | ≥19 | ≤14 | 15-18 |
| **Doxycycline** | S/18 mm | ≥16 | ≤12 | 13-15 |
| **Clindamycin** | S/26 mm | ≥21 | ≤14 | 15-20 |
| **Streptomycin** | R/8 mm | ≥15 | ≤11 | 12-14 |
| **Gentamicin** | R/0 mm | ≥15 | ≤12 | 13-14 |
| **Vancomycin** | R/9 mm | ≥17 | - | - |
| **Chloramphenicol** | S/23mm | ≥18 | ≤12 | 13-17 |
| **Source** | wound infection | | | |

# Appendix

**Table(1-6) *S. epidermidis* sensitivity test and source**

| Antibiotics | *S. epidermidis* inhibitory zone (mm) | Zone Diameter (mm) Interpretive Criteria(CLSI 2019) | | |
|---|---|---|---|---|
| | | S | R | I |
| **Ciprofloxacin** | R/0 mm | ≥21 | ≤15 | 16-20 |
| **Ofloxacin** | R/0 mm | ≥18 | ≤14 | 15-17 |
| **Levofloxacin** | R/0 mm | ≥19 | ≤15 | 16-18 |
| **Azithromycin** | R/9 mm | ≥18 | ≤13 | 14-17 |
| **Clarithromycin** | R/11 mm | ≥18 | ≤13 | 14-17 |
| **Tetracycline** | R/14 mm | ≥19 | ≤14 | 15-18 |
| **Doxycycline** | R/12 mm | ≥16 | ≤12 | 13-15 |
| **Clindamycin** | S/21 mm | ≥21 | ≤14 | 15-20 |
| **Streptomycin** | S/20 mm | ≥15 | ≤11 | 12-14 |
| **Gentamicin** | R/0 mm | ≥15 | ≤12 | 13-14 |
| **Vancomycin** | R/14 mm | ≥17 | - | - |
| **Chloramphenicol** | S/23 mm | ≥18 | ≤12 | 13-17 |
| **Source** | wound infection | | | |

# الخلاصة

خلال الفترة من تشرين الثاني 2019 إلى كانون الثاني 2020، تم جمع مائتي عينة سريرية (مسحة للجروح والحروق وعينات بول) من المرضى الراقدين في مستشفى بعقوبة التعليمي ومستشفى البتول في مدينة بعقوبة. تم زراعة العينات على أوساط اكار الدم وماكونكي. وشخصت العزلات البكتيرية باستخدام واساط انتقائية وتفريقية. ثم أجريت اختبارات البايو كيميائية في تأكيد تشخـيص كل من العزلات البكتيرية. اعتمادا على نتائج التشخيصات البايو كيميائية ، الانواع البكتيرية ظهرت كما يلي:

*Klebsiella pneumoniae*, *Escherichia coli, Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and Staphylococcus aureus.

تم اختبار الحساسية لمضادات الميكروبات لجميع العزلات البكتيرية المختلفة نحو 29 عامل للميكروبات باستخدام طريقة الانتشار القرصي وأظهرت النتائج أن العديد من العزلات البكتيرية كانت متعددة المقاومة للمضادات الحياتية (MDR).

تكتسب طريقة التخليق الحيوي للجسيمات النانوية مجالًا مهمًا للغاية نظرًا لفوائدها الاقتصادية والصديقة للبيئة. في الدراسة الحالية، استخدم الراشح الخالي من الخلايا (الشرش) من *lactobacillus* spp. في تخليق الجزئيات النانوية. بكتيريا *lactobacillus* spp. تم عزلها من منتجات الالبان المحلية وتحديدها على انها *lactobacillus* spp. باستخدام وسط زراعة الإثراء الانتقائي De Man, Rogosa and Sharpe agar (MRS agar). التشخيص المظهري والمجهري والاختبارات البايو كيميائية التي أجريت لتأكيد التشخيص. استخدم الراشح الخالي من الخلايا (الشرش) في التخليق الحيوي للجزئيات النانوية الفضية.

تم توصيف جسيمات الفضة النانوية باستخدام تحليل حيود الأشعة السينية(XRD) الذي يؤكد أن الجسيمات النانوية عبارة عن أنواع مكعبة من الفضة وكلوريد الفضة . معادلة المجالات (البلورية) ، اظهرت اقوى ثلاث قمم، كان حجم الجسيمات المحسوب 17.1 و18.6 نانومتر للفضة و24.3 نانومتر لكلوريد الفضة.

استخدم مجهر القوة الذرية AFM في توصيف الحجم والتضاريس وتوزيع حجم الحبيبات للجسيمات النانوية المُصنَّعة حيوياًAg/AgCl NPs ،حيث كان معدل الحجم لجسيمات الفضة وكلوريد الفضة النانوية 50 نانوميتر. كشف التحليل الطيفي المرئي للأشعة فوق البنفسجية(UV-Vis) عن تكوين جزئيات الفضة النانوية. اظهر التحليل الطيفي للأشعة تحت الحمراء(FTIR) مجموعات وظيفية مختلفة من الجسيمات الحيويةُ المسؤولة عن عملية التخفيض والسد. تم استخدام المجهر الإلكتروني (SEM) لتوصيف شكل وحجم الجسيمات النانوية.

فعالية جسيمات الفضة وكلوريد الفضة النانوية ضد الأنواع البكتيرية المتعددة المقاومة التي سبق اختيارها تم تحديدها عن طريق الانتشار بالحفر الاكار، لوحظ ان نمو البكتيريا قد تم تثبيط بتركيز 12,500 مايكروغرام/مللتر من جسيمات الفضة وكلوريد الفضة النانوية . كانت تراكيز الجسيمات النانوية (12,500 ; 25,000 ; 50,000 ; 100,000 ميكروغرام / مل) لها أقصى تأثير مثبط ضد بكتريا *S. aureus* (24 ، 28 ، 30 ، 32 ملم) على التوالي وأقل تأثير مثبط ضد بكتيريا *E. coli* (16 ، 21 ، 23 ، 27 ملم) على التوالي بينما تباينت التأثيرات المثبطة للبكتيريا الأخرى (*P. mirabilis* و *S. epidermidis* و *K. pneumoniae* و *P. aeruginosa*) بين البكتيريتين.

أخيرًا ، تم تحديدMIC للجسيمات النانوية Ag / AgCl بواسطة طريقة التخفيف الدقيق. أظهرت النتائج أن MIC لـ Ag / AgCl للأنواع البكتيرية *P. aeruginosa* *P. mirabilis* *S .aureus,S. epidermidis* كان 2500 مايكرو غرام /مللتر بينما MIC للأنواع البكتيرية *E. coli* و *K. pneumonia* كان *5000* مايكرو غرام /مللتر.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية العلوم


# التأثير التثبيطي للجسيمات الفضة وكلوريد الفضة النانوية المنتجة من بكتيريا *Lactobacillus* spp. ضد البكتيريا المرضية

رسالة مقدمة إلى

مجلس كلية العلوم _ جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل الطالب

**عبد الله سعد صعب الحمداني**

بكالوريوس علوم الحياة / كلية العلوم / جامعة ديالى

2017-2016

بإشراف

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