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IMMUNOLOGICAL AND MOLECULAR DETECTION OF BIOFILM FORMATION AND ANTIBIOTIC RESISTANCE GENES OF ESCHERICHIA COLI ISOLATED FROM URINARY TRACT

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ABSTRACT
Urinary tract infections (UTIs) caused by Escherichia coli are among the most prevalent bacterial infections worldwide, often associated with biofilm formation and multidrug resistance, which complicate treatment strategies. This study aimed to investigate the immunological and molecular characteristics of E. coli isolates from patients with UTIs, focusing on their ability to form biofilms and their possession of antibiotic resistance genes. A total of 140 urine samples were collected from patients in three major hospitals. Biofilm formation was assessed phenotypically using the tissue culture plate (TCP) method and Congo Red Agar (CRA), while molecular detection of biofilm-related and resistance genes was carried out using PCR. The study found a high prevalence of biofilm-forming isolates, with significant correlation to the presence of genes such as fimH, papC, and blaTEM. Moreover, antimicrobial susceptibility testing revealed high levels of resistance to commonly used antibiotics, including ampicillin, trimethoprim-sulfamethoxazole, and ciprofloxacin. These findings highlight the urgent need for improved diagnostic tools and targeted therapeutic approaches to manage biofilm-associated, multidrug-resistant E. coli infections.

KEYWORDS: Escherichia coli, urinary tract infection, biofilm formation, antibiotic resistance, PCR, fimH gene, blaTEM, immunological detection, multidrug resistance, Congo Red Agar.

INTRODUCTION

Biofilms are complex microbial communities that form on various surfaces, including medical devices and host tissues. They are embedded in a self-produced extracellular matrix composed of proteins, polysaccharides, and extracellular DNA, which provides structural support and protection. Biofilms are notoriously resistant to antibiotics and immune responses, contributing to chronic infections such as pneumonia, meningitis, and urinary tract infections (UTIs). Biofilm-related infections are classified as either device-related or tissue-related, depending on whether medical implants are involved.

Pseudomonas aeruginosa and Escherichia coli are prominent biofilm-forming pathogens in hospital-acquired infections. The extracellular matrix limits antibiotic penetration, reducing the effectiveness of treatments. The penetration efficiency of antibiotics varies by type and bacterial species. Sub-lethal antibiotic exposure can alter bacterial morphology, biofilm structure, and virulence factor expression. Moreover, some antibiotics may trigger inflammatory responses by releasing endotoxins, which complicate treatment outcomes.

The study focuses on urinary tract infections caused by Gram-negative bacteria, particularly P. aeruginosa and E. coli. It aims to analyze the presence of key virulence genes and evaluate antibiotic effects on biofilm structure and bacterial morphology.

Research Objectives

1. To isolate and identify P. aeruginosa and E. coli from clinical UTI samples.
2. To detect phenotypic virulence factors such as biofilm formation, capsule production, and hemolysis.

3. To assess the antibiotic susceptibility of the isolated strains.
4. To evaluate the antibacterial effects on biofilms during logarithmic growth.
5. To identify genotypic virulence markers (e.g., tet(M), Aph(3)-IIIa, Par-c, aac(6´)-Ib-cr) and biofilm-associated genes using PCR.

2: Literature Review

2-1: History

2-1-1. Escherichia coli (E. coli)

E. coli is a gram-negative, facultative anaerobic rod-shaped bacterium from the Enterobacteriaceae family (Joklik et al., 1992). Typically found in the intestinal tract of humans and animals, it can cause extra-intestinal infections when it invades other body sites (Sharma et al., 2007). E. coli expresses numerous virulence factors including enterotoxins, hemolysins, fimbriae, and colicins—bactericidal proteins encoded on plasmids (Mahon et al., 2002; Gillespie et al., 2000). Its strains can cause various infections like septicemia, pneumonia, meningitis, UTIs, and diarrheal diseases which are a major cause of infant mortality globally (Levine et al., 1986; Holt and Krieg, 1994; Kausar et al., 2009).

2-1-2. Pseudomonas aeruginosa

This gram-negative bacillus, equipped with a polar flagellum, can grow in a wide temperature range (Kiska and Gilligan, 2003). It produces virulence factors like pili, elastase, pyocyanin pigment, and biofilm-forming alginate (Forbes et al., 2007; Wozniak et al., 2003). Infections include septicemia, UTIs, and GI tract infections in immunocompromised hosts (Pier and Ramphal, 2005; DaSilva et al., 2004). The infection involves attachment, invasion, and systemic dissemination (Davinic, 2008).

2-2: Virulence Factors

Pathogens rely on virulence factors to colonize, evade host defenses, and spread. Many are acquired via horizontal gene transfer (Hacker and Carniel, 2001; Nasser et al., 2014). Their expression depends on environmental signals (Monack et al., 2004; Blaser and Kirschner, 2007).

2-2-1. Hemolysin Production

Hemolysins lyse erythrocytes and other immune cells, aiding immune evasion and dissemination (Farmer et al., 1989; Kayser et al., 2005; Gerald et al., 2012).

2-2-2. Protease Production

Proteases degrade host proteins and immune components, supporting invasion. Bacteria produce metalloproteases, serine, and cysteine proteases (Barrett et al., 2003; Reed, 2007).

2-2-3. Capsule Formation

Capsules of polysaccharides shield bacteria from phagocytosis and antibiotics (Schwan et al., 2005; Rajesh and Rutten, 2004).

2-2-4. Bacterial Adhesion

Adhesins interact with host cell receptors to initiate colonization. Adhesion involves pili, flagella, and LPS (Pieroni et al., 1988; Al-Dulaymi, 2003).

2-2-5. Fimbriae

Fimbriae (e.g., P fimbriae) facilitate adhesion to uroepithelial cells via glycosphingolipid receptors (Leffler and Svanborg, 1981).

2-2-6. Flagella

Flagella enhance motility and virulence, supporting bacterial spread in host environments (Chang et al., 2007; Prakash et al., 2005).

2-2-7. Siderophores

Iron-scavenging siderophores like enterobactin and yersiniabactin help bacteria thrive under iron limitation and enhance virulence (Schaible and Kaufmann, 2004; Henderson et al., 2009).

2-2-8. Endotoxins

LPS (especially lipid A) from gram-negative bacteria causes inflammatory responses, and endotoxin release increases upon cell death or antibiotic exposure (Lepper et al., 2002; Michie et al., 1988; Mathison et al., 1992).

2-2-8.1: Release of toxin after publicity to antibiotics

Above their primary bactericidal and bacteriostatic activity, antibiotics had been shown to exert other effects that can be of paramountcy for treatment of infections. One of these is the effect on the liberation of bacterial pollution.

2-2-8-2: Antibiotic-brought about release of endotoxin from gram-poor micro organism

A number of in vitro and in vivo studies have proven an incrementation in endotoxin release after exposure to extraordinary antibiotics (Lepper et al., 2002). In standard, bactericidal antibiotics liberate initially greater endotoxin than bacteriostatic antibiotics and antibiotics energetic on the cellular wall, inclusive of Penicillins and Cephalosporins, relinquish extra than antibiotics with other modes of motion, including protein synthesis inhibitors. However, there are huge versions between distinct antibiotics and even a number of the Beta -lactam antibiotics, there are awesome differences inside the propensity to loose endotoxin (Van den Berg et al., 1992). Penicillin-binding proteins (PBP) are enzymes that are placed in the bacterial mobile wall and accountable for the cellular wall synthesis. They are additionally the primary targets for the Beta -lactam antibiotics and, depending at the affinity to those PBPs, various amounts of endotoxin are liberated from the gram terrible bacteria at exposure to these antibiotics (Maskin et al., 2002). Beta-lactam antibiotics with affinity for PBP 1, lead to speedy killing with out supplemental launch of endotoxin whereas antibiotics with selective affinity for PBP 2, result in conversion of the micro organism to round cells, spheroplasts, with

lack of viability but with out cell wall ravagement and exorbitant endotoxin release. Binding to PBP three, causes selective inhibition of septation and perpetuating bacterial elongation with formation of lengthy filaments and a subsequent incremented endotoxin engenderment. Thus, release of high quantities of endotoxin is mainly related to PBP 3 binding. Cefuroxime, Cefotaxime, Piperacillin and Aztreonam bind to PBP 3 and are related to antibiotic-induced endotoxin launch (Prins van et al., 1994). Ceftazidime, at high concentrations, binds to PBP 1 and the carbapenems to PBP 2 (Gilbert et al, 2000). At decrease concentrations, Ceftazidime and Meropenem bind predominantly to PBP3 ensuing in better relinquishment of endotoxin at lower than at better doses (Mascini et al., 2001). Simultaneous inhibition of PBP 1a and three, that can be caused by Ceftazidime at mid-concentration tiers, has withal been verified to result in formation of spheroplasts. These findings have been expounded because the sum of inhibitory effects (Hansen, 2001). Among the bactericidal antibiotics, aminoglycosides inhibit protein synthesis by using binding to 16S rRNA (Gilbert, 2000).), which results in speedy killing with out extortionate endotoxin launch (Dofferhoff et al., 1991).

2-2-11: Biofilm Formation

Biofilm Definition and Formation

Biofilms are communities of bacteria encased in a self-produced EPS matrix, developing in stages from initial attachment to mature biofilm and cell release (Engel, 2003; Nickel and Costeron, 1993).

2-2-11-1: Importance of Biofilms

Biofilms contribute to chronic infections and contamination of medical devices. They are resistant to antibiotics and immune clearance (Costerton et al., 1999; Kokare et al., 2009).

2-2-11-3: Biofilm Structure – EPS

EPS makes up most of the biofilm matrix, consisting of hydrated polysaccharides and proteins. It supports stability, adhesion, and resistance (Flemming et al., 2000; Sutherland, 2001).

2-2-11-5: Correlation with Antibiotic Resistance

Biofilms impede antibiotic penetration and protect dormant bacteria. MDR strains like K. pneumoniae form strong biofilms, showing resistance to multiple antibiotics (Yang and Zhang, 2008; Subramanian et al., 2012; Sanchez et al., 2013; Hennequin et al., 2012).

2-2-12: Antibiotic Resistance

Antibiotic resistance is the ability of microorganisms to withstand antibiotics. It evolves via natural selection or can be engineered by stress-induced gene development. Resistant bacteria can spread genes horizontally through plasmids. When carrying multiple resistance genes, they're termed multi-resistant or superbugs. Resistance may be **intrinsic** (natural traits like lack of target site) or **acquired** (mutation or gene acquisition). It is also classified as **phenotypic** (temporary states like L-forms or persisters) or **genotypic** (chromosomal/plasmid-borne genes) [(Ellerbroek et al., 2004); (Inglis, 2003); (Mike et al., 2008); (Jaison, 2009)].

2-2-12-1: The Three Bacterial Lines of Defense against Antimicrobial Agents

2-2-12-1-A: The First Line of Defense: Bacterial Biofilms

Biofilms are communities of bacteria encased in EPS (extracellular polymeric substances) adhered to surfaces [(Prakash et al., 2003); (Hall-Stoodley et al., 2014); (Flemming, 2002, 2010)].

2-2-12-1-A-1: Restricted Penetration of Antimicrobial Agents

EPS can hinder antibiotic penetration via various interactions, though full blockage is rare [(Sutherland et al., 2001); (Abdallah et al., 2014); (Drenkard, 2003)]. Some antibiotics penetrate well (e.g., Vancomycin), while others face barriers depending on biofilm thickness and composition [(Dunne et al., 1993); (Pibalpakdee et al., 2012)].

2-2-12-1-A-3: Persistence

Persisters are non-growing subpopulations that survive antibiotic exposure due to altered transcription [(Qu et al., 2010); (Brooun et al., 2000); (Lewis, 2008)].

2-2-12-1-B: The Second Line of Defense: Cell Envelope and Efflux Mechanisms

2-2-12-1-B-1: Cell Wall

Resistance to glycopeptides and β-lactams results from altered precursors or PBPs and β-lactamase production [(Huang et al., 2008); (Bush, 2013); (Cetinkaya et al., 2000); (Walsh et al., 2000); (Zapun et al., 2008)].

2-2-12-1-B-2: Cell Membrane

The outer membrane restricts antibiotic entry through porins; changes in OMPs confer resistance [(Bayer et al., 2013); (Lambert, 2002); (Brözel et al., 1994); (Chapman et al., 1998); (Zhou, 2014)].

2-2-12-1-B-3: Multi-Drug Efflux Pumps

Efflux pumps (ABC, RND, etc.) eject a broad range of antibiotics, conferring MDR [(Paulsen, 2003); (Blair et al., 2014); (Handzlik et al., 2013); (Putman et al., 2000)]. The AcrAB/TolC system in E. coli is a model example [(Yamaguchi et al., 2015); (Janganan et al., 2011); (Zgurskaya et al., 2015)]. Specific systems in P. aeruginosa (MexAB-OprM, MexXY-OprM) extrude particular drugs [(Poole, 2013); (Morita et al., 2012); (Jeannot et al., 2008)].

Efflux pump inhibitors (natural and synthetic) show promise in restoring antibiotic efficacy [(Whalen et al., 2015); (Dwivedi et al., 2015)]. Efflux pumps also support biofilm resistance [(Soto, 2013); (Zhang et al., 2008); (Buroni et al., 2014); (Vikram et al., 2015)].

2-2-12-1-C: The Third Line of Defense: Intracellular Alteration

Even if antibiotics penetrate, bacteria resist through altering targets or expressing resistance genes.

2-2-12-1-C-3: Quorum Sensing (QS) Systems

QS enables population-level gene expression changes in response to antibiotics. Systems like LasR/I and RhlR/I regulate biofilm and virulence factor genes [(Miller et al., 2001); (Solano et al., 2014); (Suga et al., 2003); (Garcia-Contreras et al., 2015); (Schuster et al., 2006); (Davies et al., 1998); (Shih et al., 2002); (Brackman et al., 2011)].

2-2-12-1-C-4: Genetic Regulation

2-2-12-1-C-4-1: DNA Synthesis

Quinolone resistance is linked to mutations in gyrA and parC genes [(Drlica et al., 1997); (Jacoby, 2005)].

2-2-12-1-C-4-2: Plasmids

Plasmids carry genes encoding enzymes like β-lactamases, methylases (ermC, cfr), and resistance proteins (e.g., qnr) [(Elufisan et al., 2012); (Novick, 1986); (Foster, 1983); (Kümmerle et al., 1996); (Lina et al., 2006); (Liu et al., 2002); (Vetting et al., 2011); (Da Re et al., 2009); (Dolejska et al., 2013)].

2-2-12-1-C-4-3: Chromosome

Chromosomal mutations also contribute. SOS responses and transposons (e.g., Tn1546, Tn916) promote gene exchange and resistance [(Woodford et al., 2007); (Dorr et al., 2009); (Beaber et al., 2004); (Pray, 2008); (Arthur et al., 1993); (Garnier et al., 2000)]. Integrons mediate resistance by capturing gene cassettes [(Gillings, 2014); (Corrêa et al., 2014); (Huang et al., 2015); (Tribuddharat et al., 1999); (Hocquet et al., 2012)].

The resistome includes all resistance genes and precursors in pathogenic and non-pathogenic bacteria [(Perry et al., 2014)]. Studies using mutant libraries identified specific genes linked to resistance in P. aeruginosa and E. coli [(Fernández et al., 2013); (Breidenstein et al., 2008)].

3- MATERIALS AND METHODS

3-1:Materials

3-1-1. Equipments and instruments: As in table (3-1)

Table (3-1): Instruments and tools used in this study.

Equipments & Instruments	Manufacturing company	Origin
Autoclave	Tripod	UK
Burner	Amal	Turkey
Centrifuge	Memmert	Germany
Deep freezer	GFL	Germany
Digital camera	Sonyo	Japan
Electric Oven	Binder	Germany
Electrophoresis	Bio- Rad	Italy
ELISA System	Biotek	U.S.A
Eppendorf tubes	Sterellin Ltd	UK
Incubator	Selecta	Spain
Inoculating Loop	-	Japan
Light Microscope	Olympus	Japan
Micropipette 20-1000ul	Eppendorf	Germany
Microwave	Samsung	Korea
Millipore filter (O.22um)	Difco	USA
PCR system	Gene Amp	Singapore
PCR tubes	Eppendorf	Germany
pH meter	Orient	USA
Polystyrene 96 well plate	Eppendorf	Germany
Refrigerator	Ishtar	Iraq
Sensitive blance	Sauter	Switezeland
Spectrophotometer	Orient research	USA
shaker-incubator	Selecta	Spain
Transport swab	AFCO	Jordan
UV Lamp	UtraViolete products institute	USA
VITEK-2 compact system	BioMérieux	France
Vortex mixer	Griffin	Germany
Water bath	Gallen	Kamp
Water Distillator	Buchi	Switzerland
Vortex	Germany	Taiwan
Hood	Labogene	Danemark

3.1.2. The Biological and Chemical materials: As in table (3-2)

Table (3-2): The Biological and Chemical materials used in this study.

Chemical material	Manufacturing	Origin
Crystal Violet	Merk	Germany
Potassium chloride	Fisher	Garantie
Glucose	Fisher	Garantie
Normal saline	Pioneer	Iraq
Sodium acetate	Fluka	Switzerland
Phosphate Buffer Saline (PBS)(	BDH	England
Phosphoric acid. Bromothymol.	Geneaid	Korea
Sodium chloride, Potasium iodien,$FeCl_3$.	Fisher	Garantie
Agar-Agar	Himedia	Indian
Sodium deoxycholate.	BDH	England
Sulfuric acid.Ethanol,methanol,Glycerol	Fisher	Garantie
Hydrogen peroxide (H2O2)	SDI	Iraq
Tetramethyl p-phenyl diamine-dihydrochloride	BDH	England
Ethidium bromide	Fisher	Garantie
TBE (Tris- Borate EDTA) buffer	Fisher	Garantie
2000,100 bp DNA Ladder (DNA marker)	Fisher	Garantie
1kb DNA Ladder (DNA marker)	Fisher	Garantie
TE (Tris –EDTA)	Fisher	Garantie
Master mix	Promega	USA
Agarose	Fisher	Garantie
Blood	Blood bank	Najaf
alcohol(Ethanol)70% and 95%.	Fluka chemika	Switzerland
Congo red stain	Fisher	Garantie

3.1.3. Culture media: As in table (3-3)

Table (3-3): Culture media used in bacterial isolation.

Culture media	Manufacturing	Origin
(XLD)Xylose-Lysine medium	Oxoid	paris,France
Blood agar base	Oxoid	paris,France
Brain heart infusion broth	Oxoid	UK
Mac Conkey agar	Oxoid	paris,France
Muller-Hinton agar	Oxoid	UK
Nutrient agar	Oxoid	paris,France
Nutrient broth	Oxoid	paris,France
Salmonella Shigella agar	Oxoid	UK
Tryptic Soy broth	Himedia	India
Mannitol agar	Himedia	India
Congo red agar	Oxoid	UK

3.1.4. Prepared Kits: As in table (3-4)

Table (3-4): The Kits used in this study.

Kit type	Manufacturer	Origin
VITEK-2 AST-XN05	BioMérieux	France
VITEK-2 GN-ID	BioMérieux	France

3-1-5: Commercial kits

Table (3-5) Commercial kits used in the present study.

No.	Type of kits	Company/country
1.	DNA extraction kit	Favorgen / Taiwan
2.	Green master mix 2X Kit	Promega-USA
3.	DNA ladder 100bp	Bioneer-Korea

DNA extraction kit
Materials: FATG Buffer, FATG Buffer, FATG Column, 2 ml Collection Tube, W1 Buffer, Wash Buffer, Elution Buffer
Green master mix Kit
Materials 1- DNA polymerase enzyme (Taq). 2- dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm dTTP) 3- $MgCl_2$ (3mM) 4-Reaction buffer (pH 8.3)
DNA ladder
Materials 1-Ladder consist of 11 double-stranded DNA with size 1500 100bp. 2-Loading Dye which has a composition of (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

3-1-6: Polymerase Chain Reaction Materials: As in table (3-6)

Table (3-6): Primers used in this study.

Target Gene	Sequence	Bp	Reference
Aph(3)-llla	F 5′-**GGCTAAAATGAGAATATCACCGG** -3′ R 5′- **CTTTAAAAAATCATACAGCTCGCG** -3′	523	Vakulenko et al.,(2003)
ParC	F 5′- **TGTATGCGATGTCTGAACTG** -3′ R 5′- **CTCAATAGCAGCTCGGAATA** -3′	264	Everett et al., (1996)
Tet/tet(M)	F 5′- **GAACTGTATCCTAATGTGTG** -3′ R 5′- **GATACTCTAACCGAATCTTCG** -3′	377	Vakulenko et al.,(2003)
aac(6´)-Ib-cr	F 5′-TTGCGATGCTCTATGAGTGGCTA -3′ R 3′-CTCGAATGCCTGGCGTGTTT-5′	490	Everett et al., (1996)
Esp	F 5′- **TTGCTAATGCTAGTCCACGACC**-3′ R 5′- **GCGTCAACACTTGCATTGCCGAA**-3′	955	Vakulenko et al.,(2003)

3-2: MATERIALS AND METHODS

3-2-1: Specimen Collection

A total of 140 urine specimens were collected from UTI patients across three hospitals in Hilla (Hilla Teaching Hospital, Babylon Hospital for Maternity and Pediatric, and Mergan Teaching Hospital) between October 2021 and January 2022. Patients ranged from 6 months to 70 years old.

3-2-2: Preparation of Reagents and Solutions

Sterilization was done by autoclaving (121°C, 15 psi, 15 min). Heat-sensitive materials were filtered using 0.22 µm Millipore filters. Glassware was dry sterilized at 180–200°C for 2 hours. Solution pH was adjusted using 1M NaOH or HCl (Collee et al., 1996).

3-2-2-1: Reagents

- 3-2-2-1-1: Methyl Red: 0.1g in 300ml 95% ethanol, topped to 500ml with distilled water (MacFaddin, 2000).
- 3-2-2-1-2: Voges-Proskauer
  - Solution A: 5g α-naphthol in 100ml ethanol.
  - Solution B: 40g KOH in 100ml D.W. (Collee et al., 1996).
- 3-2-2-1-3: Oxidase: 0.1g tetramethyl-p-phenylenediamine in 10ml D.W. (MacFaddin, 2000).
- 3-2-2-1-4: Catalase: 3% H_2O_2 (MacFaddin, 2000).
- 3-2-2-1-5: Kovac's: 5g DMAB in 75ml amyl alcohol + 25ml HCl (MacFaddin, 2000).

3-2-2-2: Solutions

- 3-2-2-2-1: Normal Saline: 8.5g NaCl/L, pH 7.2 (MacFaddin, 2000).
- 3-2-2-2-2: Phosphate Buffer: 8g NaCl, 0.34g KH_2PO_4, 1.12mg K_2HPO_4 in 1L D.W., pH 7.3 (Forbes et al., 2007).
- 3-2-2-2-3: Crystal Violet (0.1%): 0.1g in 100ml D.W. (Collee et al., 1996).
- 3-2-2-2-4: Glucose: 1g in 99ml D.W., filtered (Gadeberg et al., 1983).
- 3-2-2-2-5: McFarland Standard: 0.05ml $BaCl_2$ + 9.95ml sulfuric acid (Baron et al., 1994).

3-2-3-3: Antibiotic Solutions

Prepared and filtered (Miniatis et al., 1982).

- 3-2-3-3-1: Amoxicillin: 1g in 100ml D.W. (10mg/ml).
- 3-2-3-3-2: Tetracycline: 1g in 10ml 0.1M HCl (10mg/ml).
- 3-2-3-3-3: Ciprofloxacin: 1g in 100ml D.W. (10mg/ml).

3-2-4-4: DNA and Gel Electrophoresis Solutions

- 3-2-4-4-1: TE Buffer: 0.05M Tris-OH, 0.001M EDTA, pH 8 (Promega).
- 3-2-4-4-2: TBE Buffer: 1X dilution, stored at room temp (Promega).
- 3-2-4-4-3: Ethidium Bromide: 0.05g in 10ml D.W. for DNA staining (Sambrook et al., 1989).

3-3: Preparation of Culture Media

Prepared and autoclaved as per manufacturer's instructions.

- 3-3-1: MacConkey Agar: For enteric bacteria and lactose differentiation (Winn et al., 2006).
- 3-3-2: Blood Agar: For hemolysis testing.
- 3-3-3: Mannitol Salt Agar: 111g/L (MacFaddin, 2000).
- 3-3-4: Nutrient Agar: 28g/L, general cultivation (MacFaddin, 2000).
- 3-3-5: BHI-Glycerol Broth: 5ml glycerol + 95ml BHI, for long-term storage (Collee et al., 1996; Forbes et al., 2007).
- 3-3-6: Tryptic Soy Broth: 30g/L, for biofilm testing (Collee et al., 1996).

3-4: Collection of Specimens

Samples were collected using sterile swabs, transferred to the lab, inoculated on blood, MacConkey, and MSA media, incubated at 37°C for 18–24 hrs (Collee et al., 1996).

3-4-1: Urine Specimens

Midstream urine samples were collected in sterile containers for UTI detection.

3-5: Culture of Clinical Specimens

3-5-1: Bacterial Identification

Pure colonies were identified morphologically (colony traits, Gram stain), and biochemically, then confirmed using VITEK-2 system (Collee et al., 1996).

3-5-2: Biochemical Tests (MacFaddin, 2000)

- Catalase: Gas bubbles with H_2O_2.
- Indole: Red ring with Kovac's reagent.
- Methyl Red: Bright red color indicates positive.
- VP: Red color with α-naphthol + KOH.
- KIA: Yellow for fermentation, black for H_2S.
- Motility: Diffuse growth from stab line.
- Citrate: Blue color positive.
- Urease: Pink color change.
- Gelatin: Liquefaction after refrigeration.
- Oxidase: Purple color on paper.

3-5-3: VITEK-2 Compact GN/GP ID

Used for precise bacterial ID via barcode input, card inoculation, and automated optical reading.

3-6: Preservation of Isolates

3-6-1: Short-Term

Cultured on nutrient agar/slants and stored at 4°C for up to 3 months (Harley & Prescott, 2002).

3-6-2: Long-Term

Inoculated in broth + 20% glycerol, stored at –20°C for 12–18 months (Karch et al., 1995).

3-7: Inoculum Preparation

Bacterial colonies suspended in 3ml sterile saline, adjusted to 0.5 McFarland using DensiChek (bioMérieux), used for antibiogram.

3-8: Antibiogram Profile

3-8-1: Disk Diffusion (CLSI, 2014; Bauer et al., 1966)

Tested against antibiotics (e.g., Carbenicillin, Cefuroxime, Imipenem, Ciprofloxacin, etc.) on Mueller Hinton Agar.

3-8-2: VITEK-2 AST

MIC testing with AST-XN05 and AST-N222 cards, including β-lactams, aminoglycosides, fluoroquinolones, sulfonamides, and others.

3-9: Biofilm Formation

3-9-1: Tube Method

Organisms grown in glucose-TSB, stained with crystal violet; visible lining indicates positive (Christensen et al., 1985).

3-9-2: Tissue Culture Plate Method (TCP)

Gold-standard semi-quantitative assay in 96-well plates, stained with crystal violet, OD measured at 630 nm for biofilm density (Christensen et al., 1985; Stepanovic et al., 2004).

Table (3-7): Classification of Bacterial Biofilm Formation by TCP Method.

Mean of OD value at 630 nm	Biofilm Formation
< 0.120	Non
0.120 – 0.240	Moderate
> 0.240	High

3-9-3: Congo Red Agar Method

Freeman et al. (1989) described a qualitative method for detecting biofilm formation using Congo Red Agar (CRA). The CRA medium consists of brain heart infusion broth (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo Red indicator (8 g/L). Congo Red is autoclaved separately and added at 55°C. Plates are inoculated and incubated at 37°C for 24 hrs. Biofilm production is indicated by black, dry, crystalline colonies (Reid, 1999).

3-9-4: Biofilm Examination and Measurement
Various microscopy techniques are used to study biofilm structure: light, fluorescence, DIC, TEM, SEM, AFM, and CLSM. TEM combined with polysaccharide stains like ruthenium red identifies extracellular fibers. CLSM enabled in situ examination without SEM limitations. Electron microscopy is commonly applied in medical and clinical biofilm studies (Donlan & Costerton, 2002).

3-10: Detection of Biofilm Formation with Antibiotics
The TCP method was applied with antibiotics (Ciprofloxacin, Tetracycline, Cefotaxime, Tobramycin, Norfloxacin, Amikacin, Amoxicillin, Nalidixic acid), each added (15µl of antibiotic in 10ml D.W) to wells in a 96-well polystyrene plate. The procedure followed Al-Saedi (2011), using sodium acetate for fixation.

3-11: Genomic Methods
3-11-1: DNA Extraction and Isolation
Blood agar and MacConkey-grown bacteria were cultured in broth and incubated. DNA was extracted using Favorgen kit.

A. Gram-negative bacteria
- Pellet bacteria (14,000 rpm, 1 min), discard supernatant.
- Add 200µL FATG buffer, vortex, incubate 5 min at RT.

B. Gram-positive bacteria
- Pellet, discard supernatant.
- Add 200µL lysozyme buffer, vortex, incubate 10 min, invert tube intermittently.

Lysis
- Add 200µL FABG buffer, incubate 10 min.

Binding
- Add 200µL ethanol, transfer to FABG column, centrifuge, discard flowthrough.

Washing
- Wash with W1 buffer and wash buffer, centrifuge at 14,000 rpm.

Elution
- Add 100µL preheated elution buffer or TE, centrifuge to elute DNA.

3-11-2: DNA Concentration Estimation
DNA concentration was measured spectrophotometrically at 260/280 nm. 1 OD260 = 50 µg/mL. Purity ratio (OD260/OD280) of 1.8 indicates pure DNA (Williams et al., 2007).

3-11-3: Detection of Antibiotic Resistance Genes via PCR
3-11-3-1: Primer Preparation
Lyophilized primers (Alpha DNA, Canada) were dissolved in TE buffer to prepare stock and working concentrations as per manufacturer instructions.

3-11-3-2: PCR Protocol
DNA extracts underwent PCR for resistance genes listed in Table (3-6). Reaction volume: 20 µl.

Table (3-8): Monoplex PCR Reaction Mixture.

No.	Contents of Reaction Mixture	Volume
1	Green master mix	5 µl
2	Upstream primer	2.5 µl
3	Downstream primer	2.5 µl
4	DNA template	5–7 µl
5	Nuclease-free water	3–5 µl
	Total	**20 µl**

3-11-3-3: Thermal Cycling Conditions.
Table (3-9): Thermal Cycling Conditions.

Gene Name	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
Aph(3)-IIIa	95°C / 5 min	95°C / 30s	57.8°C/30s	72°C/60s	72°C / 5 min	30
ParC	95°C / 5 min	95°C / 30s	55.2°C/30s	72°C/30s	72°C / 5 min	30
Tet/tet(M)	95°C / 5 min	95°C / 30s	54.4°C/30s	72°C/40s	72°C / 5 min	30
aac(6')-Ib-cr	94°C / 4 min	94°C / 45s	55°C / 45s	72°C/45s	72°C / 5 min	30
Esp	95°C / 5 min	95°C / 30s	61°C/30s	72°C/100s	72°C / 5 min	30

3-11-3-4: Agarose Gel Electrophoresis
The procedure followed Bartlett and Stirling (1998).

3-11-3-5: Gel Preparation and DNA Loading
Agarose (1%) in 1X TBE buffer was heated and cooled to 50°C, stained with ethidium bromide, poured into a tray, and solidified. PCR product (5µl) and DNA ladder were loaded. Electrophoresis ran at 70 volts for 1.5 hrs.

3-11-3-6: Visualization
Bands were visualized under UV using ethidium bromide staining and documented using a Biometra system.

Positive bands were confirmed when sample size matched the target gene size (Bartlett & Stirling, 1998).

Light Microscopy: Phagocytosis Estimation

Fixed smears were stained with hematoxylin (10 min), washed, then stained with eosin (30 sec), washed again, and observed under light microscopy to estimate phagocytosis activity.

3-RESULTS

3-1 : Description of study specimens

In this study a total of 140 clinical specimen were collected from burns, wound s, bone inflammation, stool and urine. The patients who attending to Hilla Teaching Hospital, the period of collection from October 2016 to January 2017.115(82.1%) gave bacterial, and the other 25(17.9%) specimens were not growth. These isolates were obtained from burns 12(10.4%), wound, 21(18.2%), bones, 12(10.4%), stool 22(19.3%) and urine 48 (41.7%). The results were shown in Figure (4-1) and table(4-1).

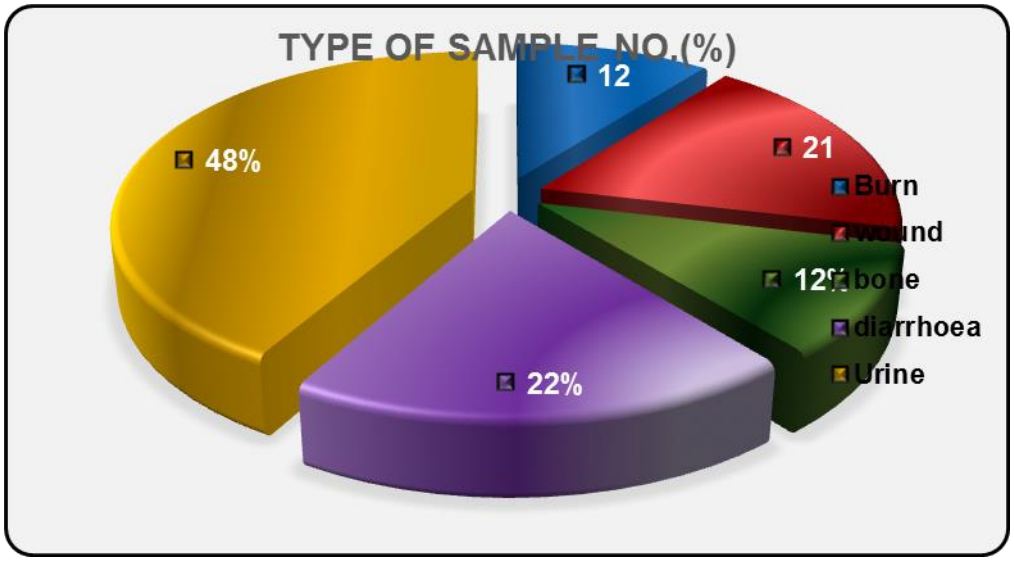


Figure (4-1): The Occurrence of Bacteria Isolated from 140 Clinical Specimen.

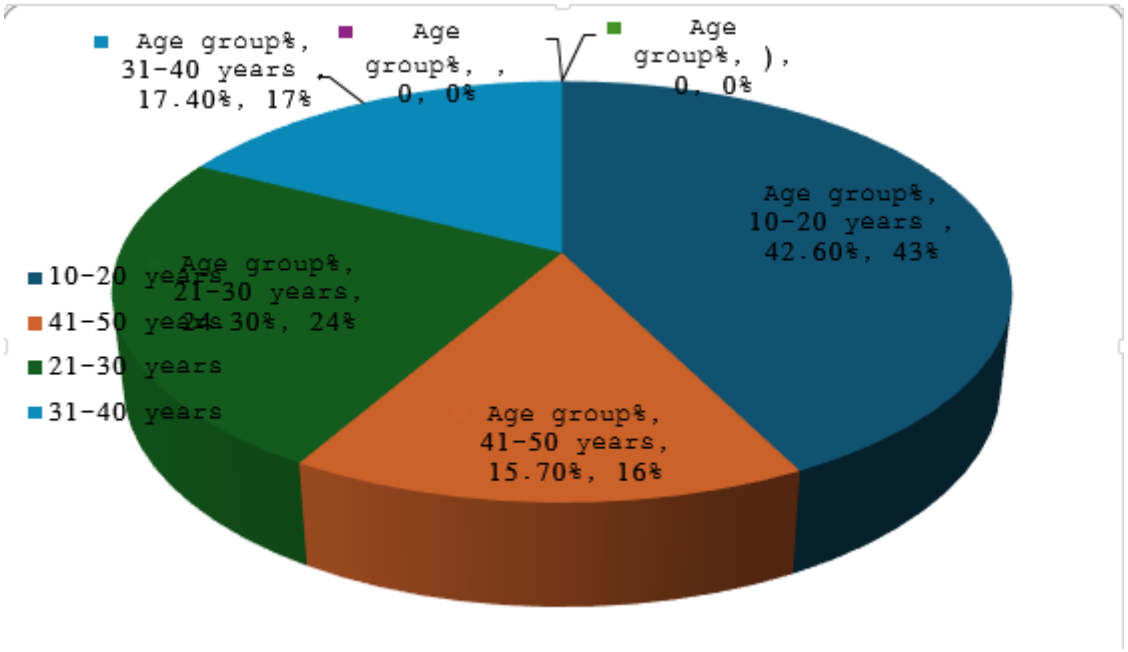
Table (4-1): Distribution of the bacteria isolated according to clinical specimen type.

Type of specimen	*No.(%)*
Burns swab	12(10.4%)
wounds swab	21(18.3%)
bones swab	12(10.4%)
Stool	22(19.2%)
Urine	48(41.7%)
Total No.(%)	115(100%)

3-1-1:Relationship between source of specimen and age and gender

3-1-1-1: Age

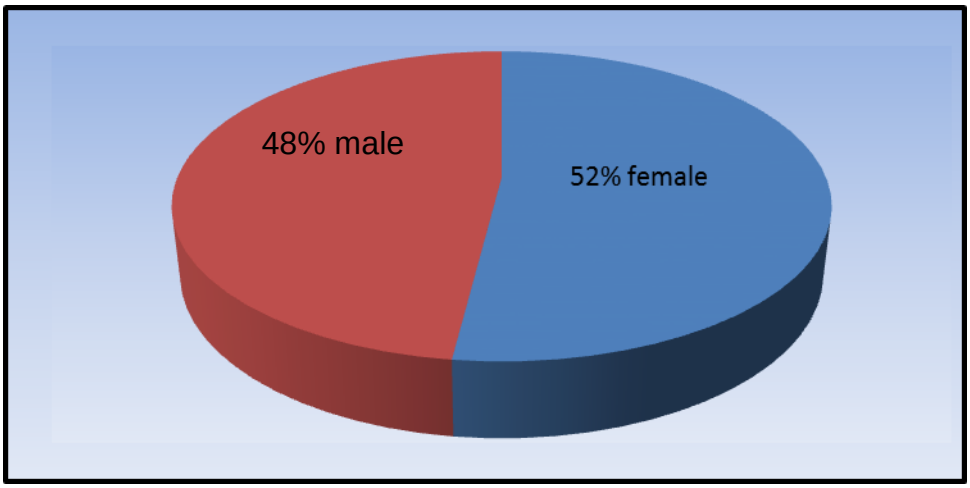
According to the patients age were found that the age group 10-20years had a highfrequency with a total 49/115 (42.6%) patients, ages 21-30years were observed to be at the second rank in the total patients which were 28/115 (24.3%), while age group (31-40),(41-50)years recorded the lowest frequency 20 /115(17.4%) and 18/115 (15.7%) patients respectively.Figure (4-2).



Figurer (3-2): Distribution of The Specimen According to the Age Groups.

3.1.1.2. Gender

The gender characteristic of patients revealed more frequency among **females were 60 (52%) than males were 55 (48%) as show in Figure (4-3).**



Figurer (4-3): Distribution of The Specimen According to Gender.

3-2: Identification of bacteria

The initially identification of bacterial specimens depended on some criteria which included Gram stain, cultural, morphology and biochemical tests. The final identification was performed with the automated vitek-2 compact system using GP, GN-ID cards which contained 64 biochemical tests and one negative control. Exactly 115 isolates were performed identification and confirmed via vitek-2 system by using four kit (GP-ID cards) two to Gram positive bacteria and(GN-ID cards) two to Gram negative bacteria.

From the 115 clinical specimen only 21(18.3%) isolates were belonged to E.coli, 18(15.6%) isolates were K. pneumonia. Out of the 115 specimens, only 26(22.6%) isolates belong to P. aeruginosa, 22(19.2%), 2(1.8%), 22(19.3%) and 4(3.5%) isolates were P. mirabilis, S.marcescens, S.aureus and S.fecalis respectively. this results were shown in Figure (4-4). In biochemical tests the results were appeared as show in table(4-2).

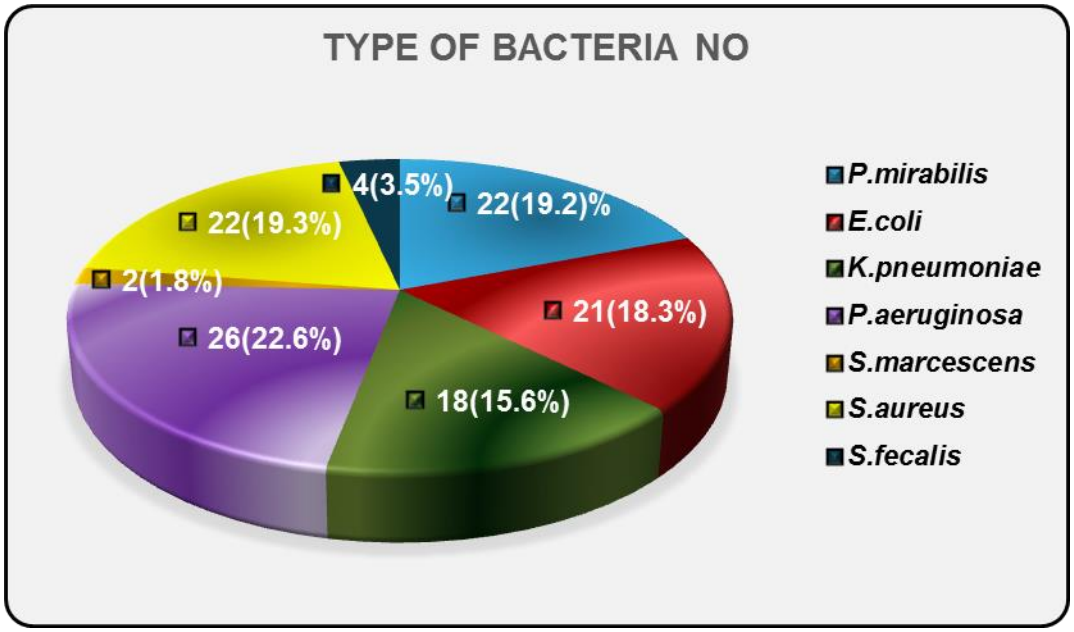


Figure (4-4): The Number and percentage of bacterial types.

Table (4-2): Conventional biochemical test.

Test / Bacteria	Oxidase	Catalase	Urease	Citrate	VP	MR	Motility	Kliglar iron agar	H2S	Indole
E. coli	-	+	-	-	-	+	+	A/A/-		+
K. pneumonia	-	+	Late +	+	+	+	-	A/A/-		+
P.aurogenosa	+	+	-	+	+	+(v)	+	Ak/Ak/-		-
P.mirabilis	-	+	+	-/+	-	+	+	Ak/A/+		-
S. marcescens	-	+	-	+	+	-	+	A/A/-		-
S.auerus.	-	+	+	+	+	+	-	-		-
S.fecalis	-	-	-	-	+	-	-	-		-

A : acid, AK : alkaline, + : positive result, - : negative result

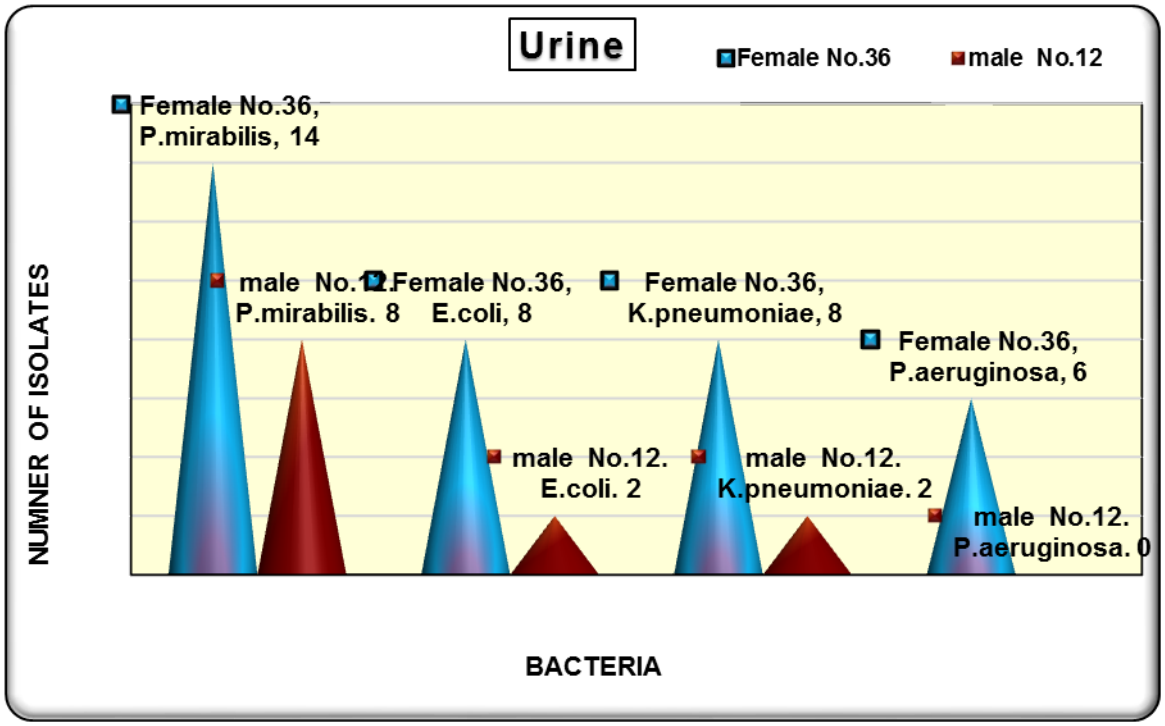


Figure (4-5) Distribution of Urines Bacterial Isolate According to The Gender.

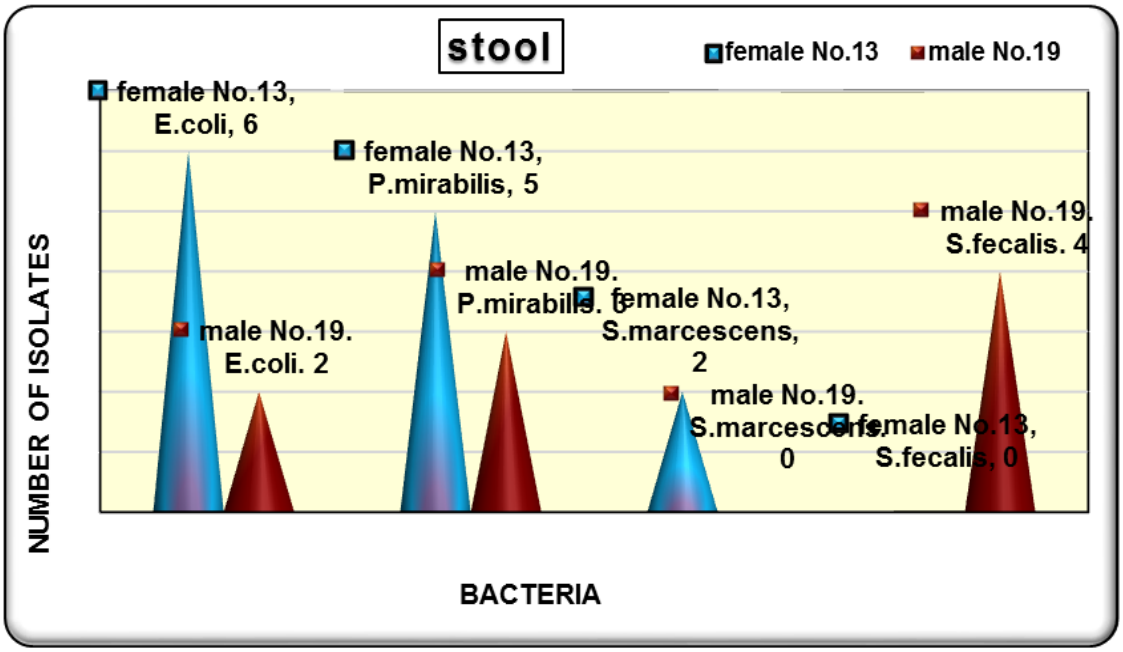


Figure (4-6) Distribution of Stool Bacterial Isolates According to The Gender.

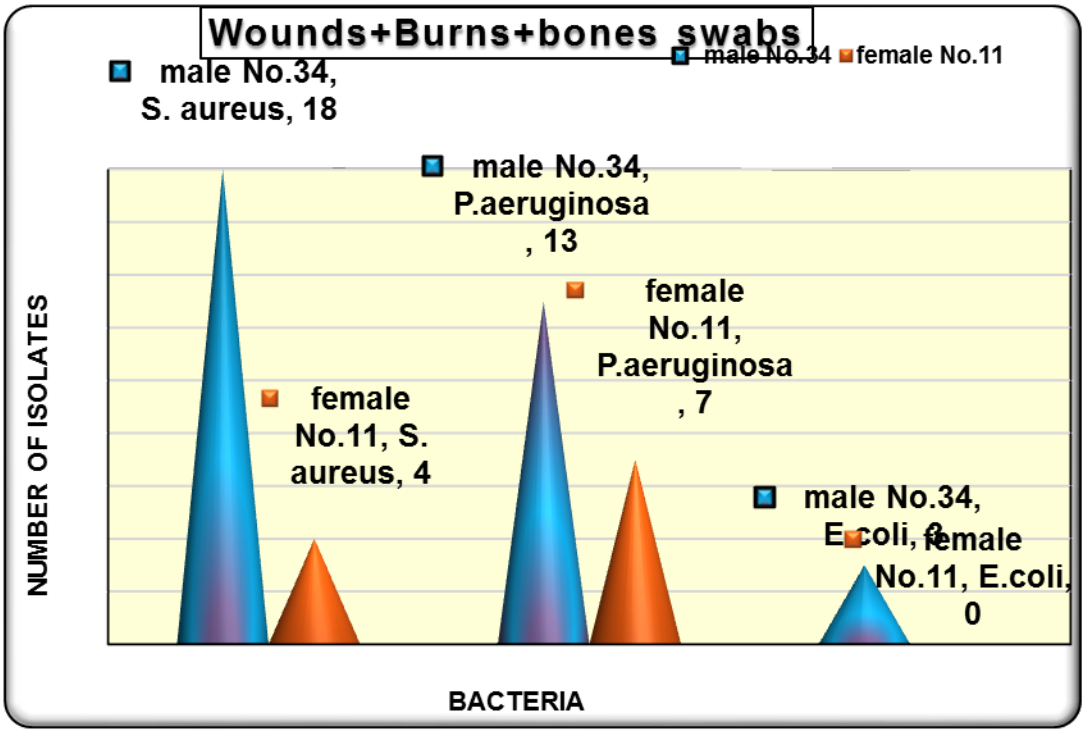


Figure (4-7) Distribution of bacteria isolated from Wounds +Burns, bones according to the gender.

Table (4-3): Distribution of the bacterial isolates according to the gender and type of.

Type of specimen / Bacteria	Burns		Wounds		Bones		Stool		Urine		*Total*
	male	Female	Male	Female	male	Female	male	Female	Male	Female	
E.coli	-	-	-	-	3	-	3	5	2	8	21
K.peumonia		-		-	-	-	2	6	2	8	18
P.aurogenosa	5	7	8	-	-	-	-	-	-	6	26
P.mirabilis	-	-	-	-	-	-	-	-	8	14	22
S. marcescens	-	-	-	-	-	-	-	2	-	-	2
S.auerus	-	-	11	2	7	2		-	-	-	22
S fecalis	-	-					4	-	-	-	4
Total	5	7	19	2	10	2	9	13	12	36	115

4-3 Detection of some virulence factors

4-3-1: Hemolysin production

A total number 115 isolates found that 75(65.2%) isolates from different types of bacteria able to produce hemolysin, results showed that E. coli 18/21(85.7%), P. aeruginosa was20/26(76.9%), S. marcescens was2/2(100%), P. mirabilis was 16/22(72.72%), and S. aureus was 19/22(86.36%) while K.pneumonia, S. fecalis they not able to produce hemolysin Table (4-4).

Table(4-4): The Number and Percentage of Hemolysin Production.

Bacteria	Hemolysin production NO.(%)	Hemolysin not production NO.(%)
E.coli	18 (85.7	3 (14.3)
K.pneumonia	0(0)	18(100)
P.aeruginosa	20 (76.9)	6(23.1)
P. mirabilis	16 (72.72)	6(27.28)
S.marcescens	2(100)	0(0)
S aureus	19 (86.36)	3(13.64)
St. fecalis	0(0)	4(100)
Total	75	40

4-3-2- Capsule Formation

A total number 115 isolates found that 23 (20%) isolates from different types of (4-5). bacteria able to capsule formation**,** results showed that E. coli was5/21(23.8%), and K.pneumonia was18/18(100%) while P. aeruginosa, S.fecalis, S**.**marcescens, P. mirabilis and S.aureus they not able to produce Capsule figure(4-8) and Table.

Table(4-5): The bacteria capsule formation.

Bacteria	Capsule not formation NO.(%)	
	Positive	Negative
E.coli	5(23.8)	16(76.2)
K.pneumonia	18(100)	0(0)
P.aeruginosa	0(0)	26(100)
P.mirabilis	0(0)	22(100)
S.marcescens	0(0)	2(100)
S aureus	0(0)	22(100)
S. fecalis	0(0)	4(100)
Total	23(20%)	92(80%)

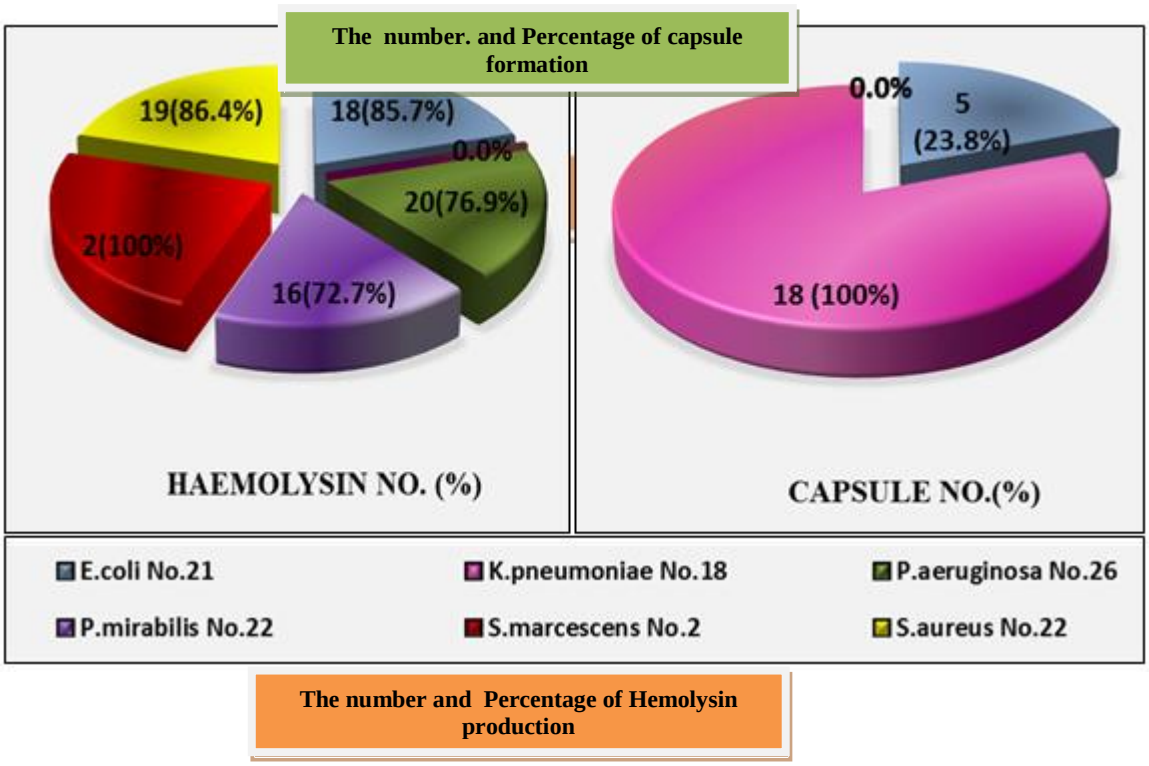


Figure (4-8): The Number and Percentage of Capsule Formation.

4-3-3: Bioflim Formation

4-3-3-1: Detection of Bioflim Formation by Tube Methods

A total number 115 isolates found that 69 isolates from different types of bacteria were tested for their able to produce biofilm as strong, 40 as moderate and 6 as weak biofilm production, results showed bioflim formation in Table (4-6) and Figure(4-9).

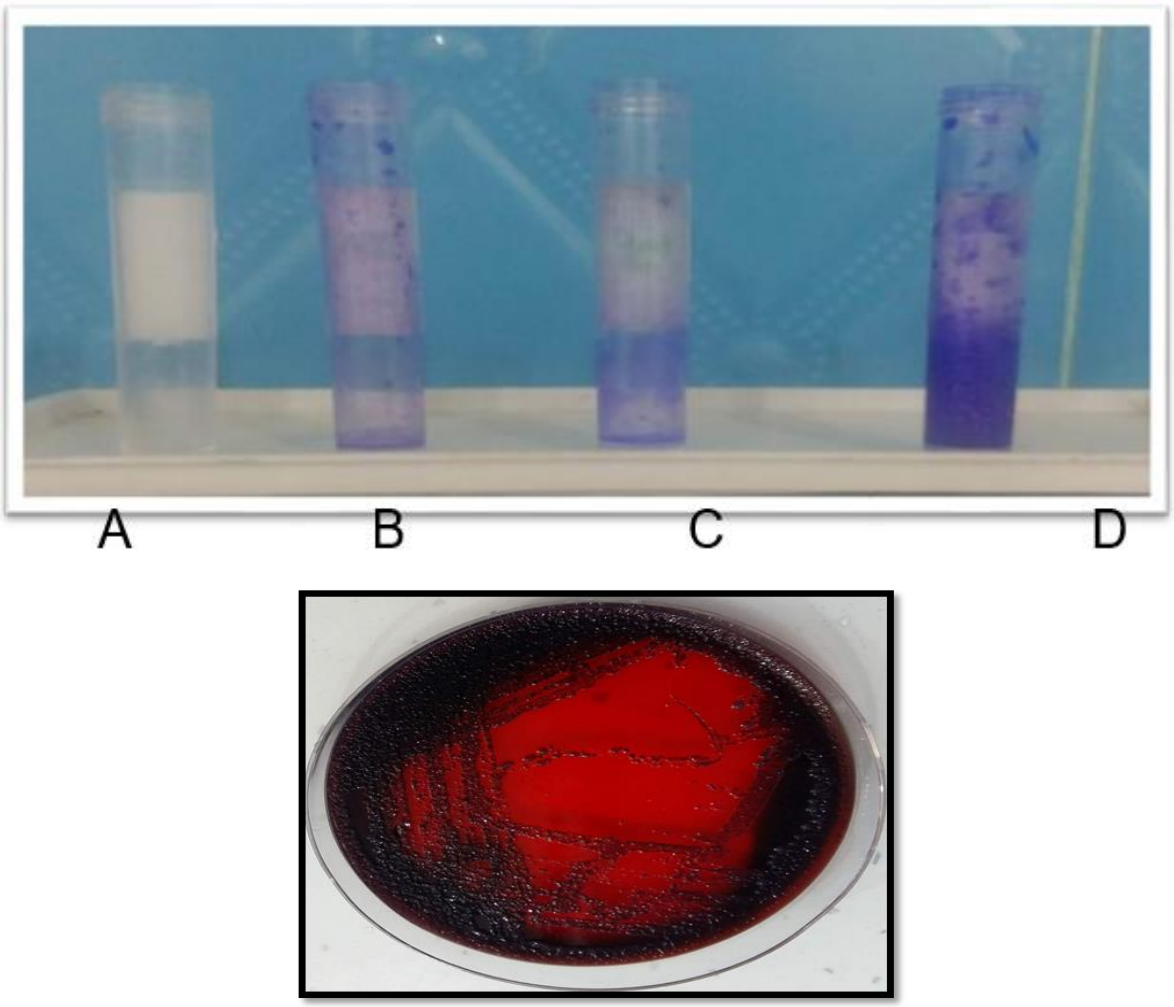


Figure (4-9): The Bioflim Formation by Tube Method Assay (TM, A-Control B-Weak C- Moderate D-Strong.

Table 4-6: The Number of Isolates Bioflim formation by Tube Method.

Types of Bacteria	Strong NO.	Moderate NO.	Weak NO.
E.coli	13	8	-
K.pneumonia	8	10	-
P.aeruginosa	13	10	3
P.mirabilis	16	6	-
S.marcescens	1	1	-
S. aureus	17	4	1
S.fecalis	1	1	2
Total	69	40	6
	115		

4-3-3-2: Detection of Bioflim Formation by Congo Red Agar

A total number 115 isolates found that 71 (61.7%)isolates from different types of bacteria able to produce bioflim by Congo Red Agar, results showed that E.coli 13/21(61.9%), P.aeruginosa was 19/26(73%), K.pneumonia16/18(88.88%), S.fecalis was1/4(25%), P.mirabilis was 13/22(59%), and S. aureus was 9/22(40.9%) while S.marcescens was negative, as in table (4-7) and Figure (4-10).

Table 4-7: The number and Percentage of Bioflim formation by congo red agar.

Bacteria	Bioflim not formation NO.(%)	
	Positive	Negative
E.coli	13(61.9)	8(38.1)
K.pneumoniae	16(88.88)	2(11.12)
P.aeruginosa	19(73)	7(27)
P. mirabilis	13(59)	9(41)
S.marcescens	0(0)	2(100)
S. aureus	9(40.9)	13(59.1)
S. fecalis	1(25)	3(75)
Total	71	44 115

4-3-3-3: Detection of Bioflim Formation by Tissue Culture Plate(TCP)

Biofilm formation on polymetric surface was tested by semi quantitative microtiter plate test (biofilm assay).This assay was repeated as triplicate for each isolate to increase the accuracy of assay (Al- Maliki, 2007).According to mean of optical density (OD) value at 630nm, the results when the mean of OD value were (>0.240,0.120 and <0.120) respectively.

Table(4-8): The number and Percentage of bioflim formation by tissue culture plat (TCP)

Bacteria	Bioflim not formation NO.(%)	
	Positive	Negative
E.coli	15(71.4)	6(28.6)
K.pneumoniae	16(88.88)	2(11.12)
P.aeruginosa	20(76.9)	6(23.1)
P. mirabilis	15(68.1)	7(31.9)
S.marcescens	1(50)	1(50)
S.aureus	13(59)	9(41)
S. fecalis	1 (25)	3(75)
Total	81	34 115

Figure (4-11): The bioflim production by tissue culture plate assay(TCP)

Table (4-9): The Comparative Between Biofilm Detection Methods.

Methods	Mean	Std. Deviation	Std. Error	Sum (%)	Sig.	LSD
Tube Method (TM))	17.83*±	7.93	3.240	107(93%)	.320**0**	8.66
Congo red agar method (CRA)	11.67	6.62	2.704	70(60.8%)		
Tissue Culture Plate (TCP)	13.33	6.47	2.642	80(69.5%)		

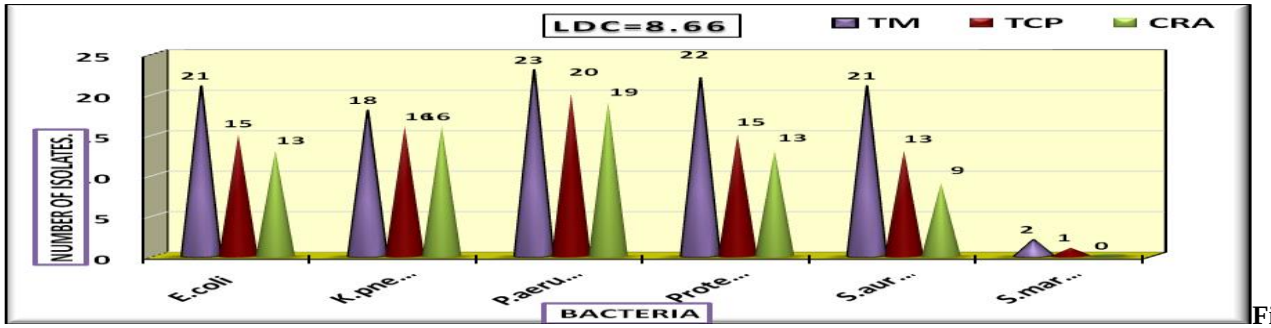


Figure (4-12): The comparative between biofilm detection methods.

Table (4-10): The number and Percentage of some virulence factors.

Bacteria	(NO. of strains)	Haemolysin production	Capsule formation	Bioflim formation		
		No. (%)	No. (%)	TM	CRA	TCP
				No. (%)	No. (%)	No. (%)
E.coli	(21)	18 (85.7)	5(23.8)	21(100)	13(61.97)	15(71.4)
K.pneumonia	(18)	0(0)	18(100)	18(100)	16(88.88)	16(88.88)
P.aeruginosa	(26)	20 (76.9)	0(0)	23(88.4)	19(73)	20(76.9)
P. mirabilis	(22)	16 (72.72)	0(0)	22(100)	13(59)	15(68.1)
S.marcescens	(2)	2(100))	0(0)	2(100)	0(0)	1(50)
S. aureus	(22)	19 (86.36)	0(0)	21(95.4)	9(40.9)	13(59)
S. fecalis	(4)	0(0)	0(0)	2(50)	1 (25)	1 (25)
	115					

Antibiotics / Bactria	Amikacin (%)	Norfloxaxin (%)	Cefotaxim (%)	Amoxicillin (%)	Tobromycin (%)	Nalidixic acid (%)	Gentemycin (%)	Tetracyclin (%)	Ciprofloxacin (%)
E.coli (21)	100	24.4	72	22.2	100	88	100	77.7	100
K.pneumonia (18)	100	94.4	72	22.2	100	88	100	77.7	100
P.aeruginosa (26)	110	96.1	30.7	11.5	92.3	76.9	92	3.8	96.1
P. mirabilis (22)	100	81	13.6	22.7	31.8	59	90.9	50.4	90.9
S.marcescens (2)	100	100	100	0	100	100	100	50%	100
S. *aureus*(22)	46	60	30	26	60	30	30	20%	70
S. fecalis(4)	100	100	100	100	0	0	66.6	66.6	100
Total									

4-4- Antibiotic sensitivity

In table (4-11), the susceptibility of 115 (E.coli, K.pneumonia, P.aeruginosa, P. mirabilis, S.marcescens, S. aureus and S. fecalis) isolates against 9 selected antibiotics was studied to determine the pattern of isolates sensitivity to various antibiotics depending on disk diffusion method.

The results represent the antibiogram profile of the isolates, indicate that isolates varied in their susceptibility to the antibiotics. The sensitivity rate of isolates to Amikacin E.coli, K.pneumonia, P.aeruginosa, P. mirabilis, S.marcescens, S. aureus and S. fecalis 100%, 100%, 100%, 100%, 100%, 46%, 100%, respectively.

The date revealed the sensitivity of E.coli to antibiotics were(24.4%), K.pneumonia 94.4%, P.aeruginosa 96.1%, P. mirabilis 81%, S.marcescens100%\, S. aureus 60% and S.fecali (100% to Norfloxacin.

The date revealed the cephalosporins sensitivity of E.coli to antibiotics were 72%,K.pneumoniae72%, P.aeruginosa 30.7%, Proteus mirabilis13.6%, S.marcescens 100%, S. aureus 30% and S.fecalis 100% of isolates being resistant to Cefotaxime.

Additionally, sensitivity of E.coli to Tobramycin antibiotics were 100%, K.pneumonia 100%, P.aeruginosa 92.3%, P. mirabilis 31.8%, S.marcescens(100%), Staphylococcus aureus 60% and S. fecalis 0% of isolates being sensitivity to Tobramycin.

In addition to the sensitivity to Gentamycin, were E.coli, K.pneumonia, P.aeruginosa, Proteus mirabilis, S.marcescens, S.aureus and S. fecalis 100%, 100, 92, 90.9, 100,30, 66.6% respectively.

Sensitivity of isolates to ciprofloxacin were 100%, 100%, 96.1%, 90.9%, 100%, 70%, 100% for E.coli, K.pneumoniae, P.aeruginosa, P.mirabilis, S.marcescens, S. aureu sand S. fecalis, respectively.

In addition to the sensitivity to Naldixic acid E.coli, K.pneumoniae, P.aeruginosa, P. mirabilis, S.marcescens, S aureus and S.fecalis 88%, 88%, 76.9%, 59%, 100%, 30%, 0%, respectively.

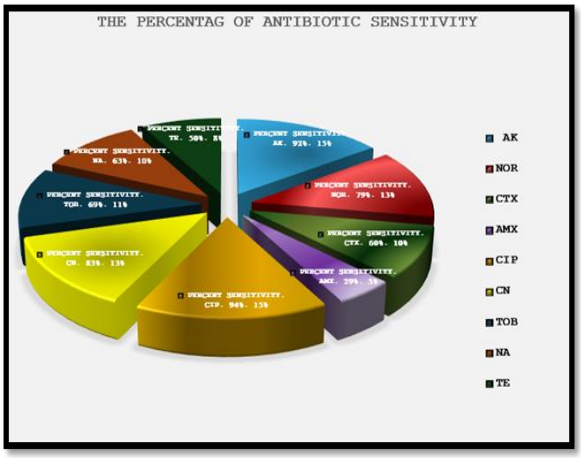


Figure.(4-13) The antibiotics sensitivity of bacterial Isolates.

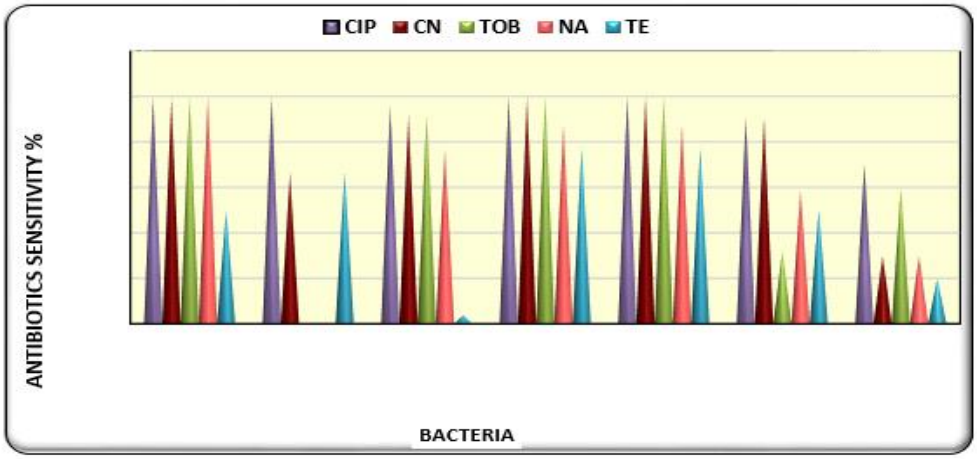


Figure.(4-14) Antibiotics susceptibility profile of bacterial isolates by disk diffusion method (CIP: Ciprofoxacin, TOB: Tobramycin, CN: Gentamicin, NA: Naldixic acid and Tet: Tetracycline) (n= 115).

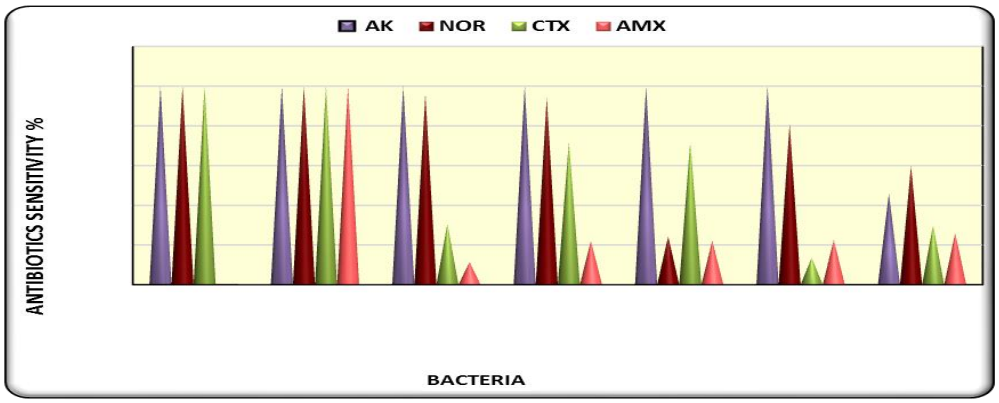


Figure.(4-15) Antibiotics susceptibility profile of bacterial isolates by disk diffusion method(AK: Amikacin, NOR : Norfloxacin, CTX: Cefotaxime and AMX: Amoxicillin) (n= 115).

The sensitivity rate of isolates to Amoxicillin E.coli, K.pneumonia, P.aeruginosa, P.mirabilis, S.marcescens, S. aureus and S.fecalis 22.2%, 22.2%, 11.5%, 22.7%, 0%, 26%, 100% respectively.

The sensitivity rate of isolates to Tetracycline E.coli, K.pneumoniae, P.aeruginosa, Proteus mirabilis, S.marcescens, S. aureus and S. fecalis(77.7%, 77.7%, 3.8%, 50.4%, 50%,20%, 66.6%), respectively.

4-4-4-1: Minimum inhibitory Concentrations (MIC) by VITEK-2 Compact

Antibiogram testing was performed with the automated VITEK-2 compact system by using AST-P580, AST-XN05 and AST-N222 cards as show on. The results showed in table(4-13) represent the antibiogram profile of bacterial isolates, indicate that isolates varied in their susceptibility to the antibiotics.

Table (4-13): Minimum Inhibitory Concentration (MIC) values of each Antibiotics for Bacterial Isolates.

Types of bacteria	**Antibiotic**	**MIC value in specimen(µg/ml)**		**Antibiotic**	**MIC value in specimen(µg/ml)**	
Staph. aureus	Benzylpenicillin	>0.25	R	+Ofloxacin		S
	Cefixime		R	Oxacillin	>2	R
	Cefoxitin Screen	POS	+	Rifampicin	>16	R
	Clindamycin	>4	R	Teicoplanin	>16	R
	Erythromycin	>4	R	Tetracycline	>8	R
	Fosfomycin	64	R	Tigecycline	1	
	Fusidic acid	>16	R	Tobramycin	2	S
	Gentamicin	>8	R	Trimethoprim/ sulfamethoxazole	80	R
	+Imipenem		R	Vancomycin	>16	R
	Inducible Clindamycin Resistance	NEG	-	+Amoxicillin/ clavulanic acid		R
	Levofloxacin	0.25	S	+Azithromyin		R
	Linezolid	>4		Ceftriaxone		R
	Moxifloxacin	0.5	S	Cefuroxime		R
	Mupirocin	>4		+Ciprofloxacin		S
	Nitrofurantion	32	S			
Types of bacteria	Antibiotic	MIC value in specimen (µg/ml)		Antibiotic	MIC value in specimen Rµg/ml)	
E. coli	Amikacin	>32	R	Minocycline	>8	S
	Aztreonam	[16]	*R	Pefloxacin	>8	R
	Cefepime	[<=1]	*R	Piperacillin	>64	R
	Ceftazidime	>32	R	Piperacillin/Tazobactam	8	S
	Ciprofloxacin	>2	R	Ticarcillin	>64	R
	Colistin	<=0.5	S	Tobramycin	8	R
	Gentamicin	>8	R	Trimethoprim/ sulfamethoxazole	>16	R
	Imipenem	<=0.25	S			
	Meropenem	<=0.25	S			
Types of	Antibiotic	MIC value in		Antibiotic	MIC value in	

bacteria		specimen (µg/ml)			specimen Rµg/ml)	
P.mirabilis	Amikacin	16	S	Minocycline	[4]	*R
	Aztreonam	>32	R	Pefloxacin	>8	R
	Cefepime	>32	R	Piperacillin	>64	R
	Ceftazidime	>32	R	Piperacillin/Tazobactam	32	I
	Ciprofloxacin	>2	R	Ticarcillin	>64	R
	Colistin	[<=0.5*]	*R	Tobramycin	>8	R
	Gentamicin	>8	R	Trimethoprim/ sulfamethoxazole	>160	R
	Imipenem	<=0.25	S			
	Meropenem	<=0.25	S			
S.marcescens	Antibiotic	MIC value in specimen (µg/ml)		Antibiotic	MIC value in specimen Rµg/ml)	
	Amikacin	<=2	S	Meropenem	<=0.25	S
	Aztreonam	<=1	S	Minocycline	8	I
	Cefepime	<=1	S	Pefloxacin	<=0.25	S
	Ceftazidime	4	S	Piperacillin	>64	R
	Ciprofloxacin	<=0.25	S	Ticarcillin	>64	R
	Colistin	[<=0.5 *]	*R	Tobramycin	<=1	S
	Gentamicin	<=1	S	Trimethoprim/ sulfamethoxazole	>160	R
P.aeruginosa	Antibiotic	MIC value in specimen (µg/ml)		Antibiotic	MIC value in specimen Rµg/ml)	
	Amikacin	<=2	S	Minocycline		*R
	Cefepime	<=1	S	Pefloxacin	0.5	S
	Ceftazidime	<=1	S	Piperacillin	<=4	S
	Ciprofloxacin	<=0.25	S	Piperacillin/Tazobactam	<=4	S
	Colistin	<=0.5	S	Ticarcillin	<=8	S
	Gentamicin	<=1	S	Ticarcillin-clavulanic acid	<=8	S
	Imipenem	<=0.25	S	Tobramycin	<=1	S
	Meropenem	<=0.25	S	Trimethoprim/ Sulfamethoxazole		*R
K.pneumoniae	Antibiotic	MIC value in specimen (µg/ml)		Antibiotic	MIC value in specimen Rµg/ml)	
	Amikacin	<=2	S	Meropenem	<=0.25	S
	Aztreonam	[16]	*R	Minocycline	8	R
	Cefepime	[2]	*R	Pefloxacin	<=0.25	S
	Ceftazidime	[4]	*R	Piperacillin	>64	R
	Ciprofloxacin	<=0.25	S	Piperacillin/Tazobactam	<=4	S
	Colistin	>8	R	Ticarcillin	>64	R
	Gentamicin	>8	R	Tobramycin	8	I
	Imipenem	1	S	Trimethoprim/ sulfamethoxazole	<=20	S
E. faecalis	Antibiotic	MIC value in specimen (µg/ml)				
	Clindamycin	>4	R			
	Erythromycin	>4	R			
	Levofloxacin	0.25	S			
	Linezolid	>4	R			
	Moxifloxacin	0.5	S			
	nitrofurantion	64	I			
	Teicoplanin	>16	R			
	Tetracycline	>8	R			
	Tigecycline	1				

	Trimethoprim/ sulfamethoxazole	80	R
	Vancomycin	>16	R

Determination of the minimum inhibitory concentrations (MICs) was done as complementary test to the previous antibiotic susceptibility test to verify resistance level of isolates towards their substrates. An isolate was characterized as resistant, if the MIC equal or greater than the breakpoint, MIC determined according to CLSI,(2014)

4-4-4-2: Effect of antibiotics on biofilm formation

Bacteria isolates biofilm formation was reduced by using some antibiotics (Tetracycline, Nalidixic acid, Cefepime, Amoxillin, Amoxi/Clavulanic acid, Ciprofloxacin, Tobramicin, Gentamicin, Amikacin). The result that revealed Significant differences at $p<0.05$ this result show in Table (4-14).

Table 4-14: Effect of antibiotics on biofilm formation.

Bacterial isolates	Biofilm formation before adding antibiotic	Biofilm formation after adding antibiotic(O.D)								
		Tet	NA	CTX	AMX	NOR	CIP	Tobr	CN	AK
E.coli	0.137	0,134	0.282	0.303	0.167	0.337	0.259	0.134	0.296	0.240
E.coli	0.083	0.116	0.078	0.230	0.163	0.133	0.492	0.132	0.142	0.075
K. pneumonia	0.196	0.127	0.249	0.316	0.332	0.291	0.957	0.314	0.133	0.240
K. pneumonia	0.124	0.097	0.214	0.209	0.088	0.185	0.108	0.071	0.126	0.137
S. marcescens	0.099	0.110	0.109	0.191	0.243	0.310	1.932	0.165	0.237	0.143
S. marcescens	0.090	0.118	0.160	0.102	0.092	0.092	1.009	0.089	0.111	0.090
P.mirabilis	0.099	0.151	0.100	0.358	0.254	0.198	0.720	0.170	0.206	0.200
P.mirabilis	0.069	0.078	0.090	0.088	0.131	0.234	0.132	0.142	0.075	0.142
P.aeruginosa	0.114	0.328	0.119	0.310	0.168	0.217	0.468	0.219	0.210	0.226
P.aeruginosa	0.103	0.343	0.081	0.233	0.116	0.141	0.397	0.155	0.126	0.178
S. aureus	0.134	0.247	0.188	0.186	0.214	0.257	0.211	0.146	0.194	0.119
S. aureus	0.090	0.090	0.182	0.137	0.208	0.208	0.769	0.221	0.075	0.135
S. fecalis	0.281	0.146	0.194	0.132	0.130	0.124	1.086	0.149	0.141	0.256
S. fecalis	0.127	0.124	0.102	0.140	0.159	0.119	0.104	0.106	0.080	0.123

Tet: Tetracyclin, NA: Nalidixiacid, CTX: Cefotaxim, AMX: Amoxicillin, NOR: Norfloxacin, CIP: Ciprofloxacin, TOB. Tobromicin, CN: Gentamicin, AK: Amikacin

4-5: Molecular detection of bacterial isolates

4-5-1: Detection of the tet(M)gene

All isolates were investigated to detect genes tet(M) which encode for enzymes responsible for catalysis Tetracycline antibiotics using PCR technique with specific forward and reverse primers.

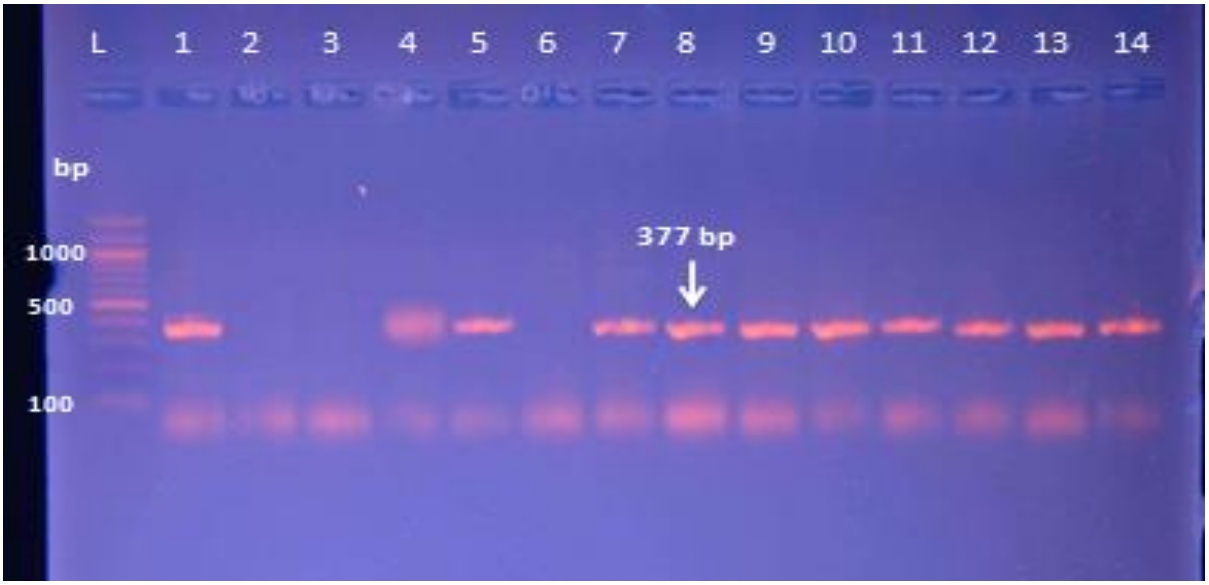


Figure. (4-22) Ethedium bromide – stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with tet(m) gene primer with product 377 bp. (1.5% agarose gel, 75 V, 1.20 hours), L:Lader, 1--E.coli, 2-E.coli, 3- K. pneumonia,43- K. pneumonia, 5- S. marcescens,6- S. marcescens, 7- Proteus mirabilis,8- Proteus mirabilis, 9-P.aeruginosa,10-9-P.aeruginosa,11- S. aureus.12- S. aureus, 13- S. fecalis, 14- S. fecalis.

4-5-2-Detection of the Aph(3)-llla gene

Aph(3)-llla genes encoding amino-glycoside modifying enzymes(AMEs) using PCR technique.

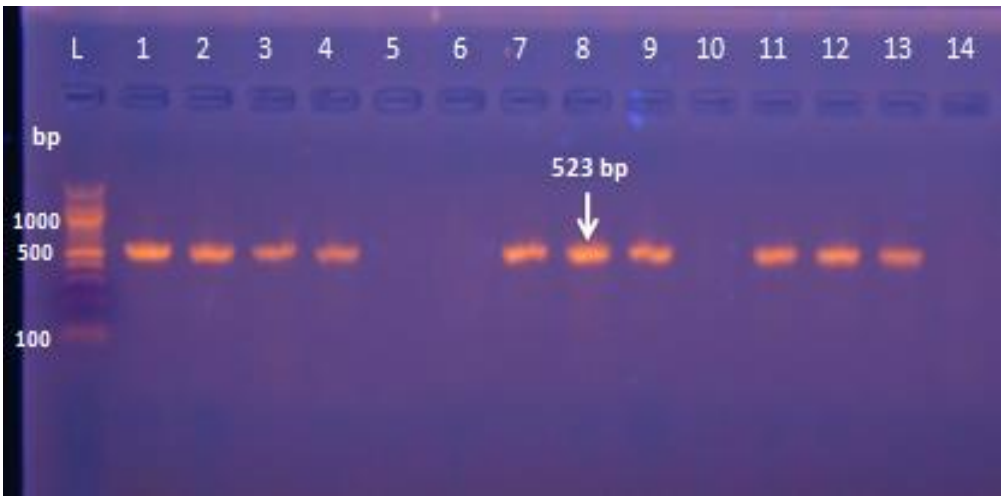


Figure. 4.23: Ethedium bromide – stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with Aph(3)-llla gene primer with product 523 bp. (1.5% agarose gel, 75 V, 1.20 hours) L Lader, E.coli, 2-E.coli, 3- K. pneumonia,43- K. pneumonia, 5- S. marcescens,6- S. marcescens, 7- Proteus mirabilis, 8- Proteus mirabilis, 9-P.aeruginosa,10-9-P.aeruginosa,11- S. aureus.12- S. aureus, 13- S. fecalis, 14- S. fecalis.

4-5 -3-Detection of the Par-c gene

Par-c gene which encode for enzymes responsible for catalysis fluoroquinolone antibiotics using PCR technique with specific forward and reverse primers. Seen from the results shown in Figure(4-24) of the current study to Par-cgene tested isolates represented 14(100%) in bacterial isolated.

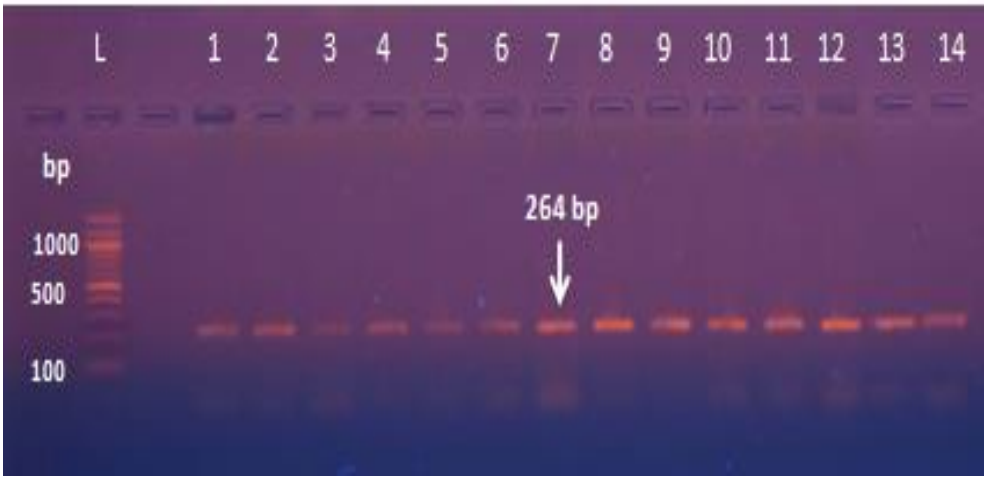


Figure. (4.24) Ethedium bromide – stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with Par-c gene primer with product 264 bp. (1.5% agarose gel, 75 V, 1.20 hours) L-Lader, --E.coli, 2-E.coli, 3- K. pneumonia,43- K. pneumonia, 5- S. marcescens,6- S. marcescens, 7- Proteus mirabilis, 8- Proteus mirabilis, 9-P.aeruginosa,10-9-P.aeruginosa,11- S. aureus.12- S. aureus, 13- S. fecalis, 14- S. fecalis.

4-5-4: Detection of the aac(6´)-Ib-cr gene

aac(6´)-Ib-cr gene which encode for enzymes responsible for catalysis plasmid -Mediated Quinolone Resistance Genes using PCR technique with PCR specific forward and reverse primers.

Figure. (2.25) Ethedium bromide – stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with aac(6´)-Ib-cr gene primer with product 490 bp. (1.5% agarose gel, 75 V, 1.20 hours) L-Lader, --E.coli, 2-E.coli, 3- K. pneumonia,43- K. pneumonia, 5- S. marcescens,6- S. marcescens, 7-., 8- P.mirabilis, 9-P.aeruginosa,10-9-P.aeruginosa,11- S. aureus.12- S. aureus,13-- S. fecalis,14- S. fecalis.

4-5-5-Detection of the esp gene

Esp gene which encode for enzymes responsible for Biofilm formation in bacterial isolates using PCR technique with specific forward and reverse primers.

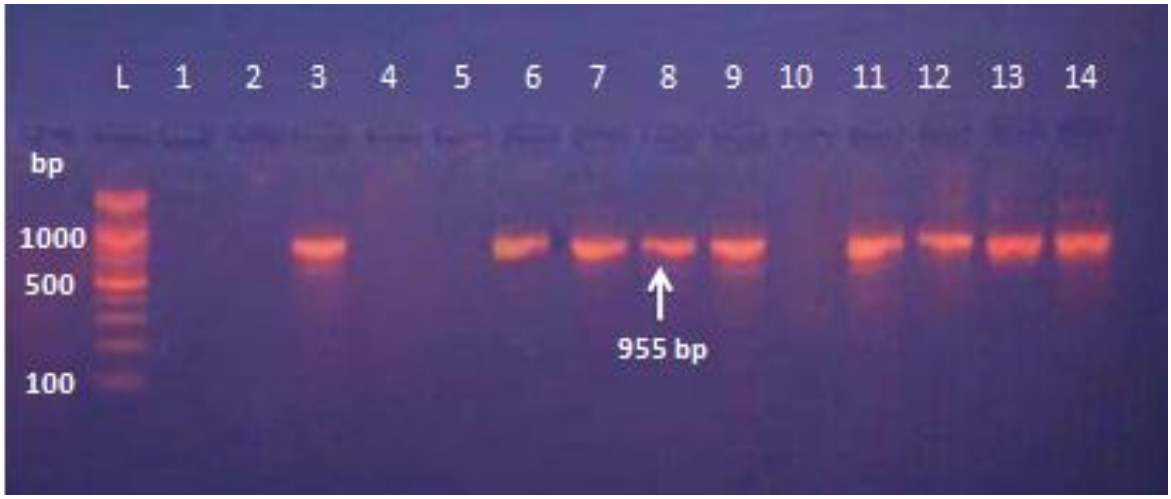


Figure. (2.26) Ethedium bromide – stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with esp gene primer with product 955 bp. (1.5% agarose gel, 75 V, 1.20 hours) L-Lader, --E.coli, 2-E.coli, 3- K. pneumonia,43- K. pneumonia, 5- S. marcescens,6- S. marcescens, 7- Proteus mirabilis,8- Proteus mirabilis, 9-P.aeruginosa,10-9-P.aeruginosa,11- S. aureus.12- S. aureus,13-13- S. fecalis,14-13- S. fecal.

Table 4-15: The comparative between types of genes.

Bacterial isolates	Biofilm by Tube method	*Esp* gene	*Antibiotic resistance*	*Antibiotic* gene resistance				Capsule formation	Haemollysin production
				Tet(m)	*Aph(3)-IIIa*	*Par-c*	*aac(6')-Ib-cr*		
E.coli	Moderate	-	NOR,AX,	+	+	+	+	+	+
E.coli	Strong	-	,TE,NA,AX	-	+	+	-	-	+
K. pneumonia	Moderate	+	,AX,CTX	-	+	+	+	+	-
K. Pneumonia	Strong	-	AX,NA,CTX	+	+	+	+	+	-
S. marcescens	Moderate	-	,AX	+	-	+	-	-	+
S. marcescens	Moderate	+	AX	+	-	+	-	-	+
P. mirabilis	Strong	+	AX.CTX.NOR ,TE	+	+	+	+	-	+
P. mirabilis	Moderate	+	NA,TE,CTX	+	+	+	-	-	+
P.aeruginosa	Strong	+	CTX.NOR,AX ,TE	+	+	+	+	-	+
P.aeruginosa	Moderate	-	TET. TOB,AX	+	-	+	+	-	+
S. aureus	Strong	+	AX.CTX.,NA, TE	+	+	+	+	-	+
S. aureus	moderate	+	AK,,CTX AX,TOB	+	+	+	+	-	-
S fecalis	moderate	+	TOB	+	+	+	+	-	-
S. fecalis	strong	+	TOB	+	-	+	+	-	-

Table 4-16: Interaction between esp gene with biofilm production by TM(tube method).

Bacterial isolates	Biofilm by Tube method	*Esp* gene	*Antibiotic resistance*	*Antibiotic* gene resistance				Capsule formation	Haemollysin production
				Tet(m)	*Aph(3)-IIIa*	*Par-c*	*aac(6´)-Ib-cr*		
E.coli	Moderate	-	NOR,AX,	+	+	+	+	+	+
E.coli	Strong	-	,TE,NA,AX	-	+	+	-	-	+
K. pneumonia	Moderate	+	,AX,CTX	-	+	+	+	+	-
K. Pneumonia	Strong	-	AX,NA,CTX	+	+	+	+	+	-
S. marcescens	Moderate	-	,AX	+	-	+	-	-	+
S. marcescens	Moderate	+	AX	+	-	+	-	-	+
P. mirabilis	Strong	+	AX.CTX.NOR ,TE	+	+	+	+	-	+
P. mirabilis	Moderate	+	NA,TE,CTX	+	+	+	-	-	+
P.aeruginosa	Strong	+	CTX.NOR,AX ,TE	+	+	+	+	-	+
P.aeruginosa	Moderate	-	TET. TOB,AX	+	-	+	+	-	+
S. aureus	Strong	+	AX.CTX.,NA, TE	+	+	+	+	-	+
S. aureus	moderate	+	AK,,CTX AX,TOB	+	+	+	+	-	-
S fecalis	moderate	+	TOB	+	+	+	+	-	-
S. fecalis	strong	+	TOB	+	-	+	+	-	-

CONCLUSIONS

In this study, Gram-positive bacterial isolates were found to be more prevalent than Gram-negative ones, with Pseudomonas aeruginosa identified as the most dominant species. The frequency of infection was higher among females compared to males. Regarding age distribution, the highest incidence was observed in the 10–20 years age group, accounting for 42.6% of the cases. All bacterial isolates demonstrated various virulence factors, including capsule formation, hemolysin production, biofilm formation, and resistance to multiple antibiotics. Ciprofloxacin exhibited the highest efficacy against the bacterial isolates, while amoxicillin showed the least effectiveness. Furthermore, strong biofilm production was confirmed using three distinct detection methods: the tube method, Congo Red Agar (CRA) method, and Tissue Culture Plate (TCP) assay. Notably, biofilm formation was significantly reduced upon treatment with several antibiotics, such as tetracycline, nalidixic acid, cefepime, amoxicillin, amoxicillin/clavulanic acid, ciprofloxacin, tobramycin, gentamicin, and amikacin—collectively considered antibiofilm agents. Molecular analysis revealed that the most frequently detected resistance and virulence-associated genes among the isolates were tet(M), Aph(3)-IIIa, ParC, aac(6´)-Ib-cr, and esp genes.

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