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The Relationship Between Some Virulence Factors and Antibiotics Resistance Determinants in Pathogenic Bacteria

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بِسمِ اللهِ الرحَمنِ الرحَيمِ

﴿يَرفَعُ اللهُ الذينَ آمنوا مِنكُمْ والذينَ أُتوا العَلمَ دَرَجاتْ واللهُ بما تَعْمَلونَ خَبير﴾

صَدَقَ اللهُ العَليُ العَظِيم

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Certification

I certify that this research *entitled* " **The Relationship Between Some Virulence Factors and Antibiotics Resistance Determinants in Pathogenic Bacteria** " was prepared under my supervision at the department of Genetic Engineering, College of Biotechnology, University of Al-Qasim Green, as a partial requirement for the degree of Bachelorism in Biotechnology.

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Dedication

"Almost every successful person begins with two beliefs: the future can be better than the present, and I have the power to make it so."

-David Brooks

I would like to dedicate my humble efforts to my homeland Iraq, the warmest womb, hopping that it would add to its successes and lead it to a bright future.

Also to my supportive and loving father and mother, whose affection, love, encouragement and continuous prayers pushed me to achieve such a success and honor.

As well as all hard working and respected teachers.

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Summary

The present study was done to scrutinize the possible relation between virulence genes and antimicrobial resistance in some pathogenic bacteria. Considering the fact that the presence of recognized infective determinants among clinical isolates may promote the emergence of infections and persistence of pathogens.

A total of 60 bacterial isolates were obtained from College of Science/ University of Al-Kufa. After the maintenance and subcultured of these isolates, some phenotype tests were performed which included plate hemolysis, haemagglutination, serum resistance and biofilm formation. Then genotype study was investigated for some virulence genes (biofilm, FimH and Iss) and antibiotics resistance genes (*bla*$_{AmpC}$, *bla*$_{TEM}$, *bla*$_{SHV\text{-}5}$ and *bla*$_{CTX\text{-}M}$) by using PCR technique.

The results of plate hemolysis indicated that all isolates of Klebsiella pneumonia and *Serratia marcescens* were positive with 100%, while *Shigella dysenteriae* and *Pseudomonas aeruginosa* isolates showed the less percentage 40% among all bacterial isolates. Haemagglutination test revealed the highest percentage with isolates of *Serratia marcescens* 100%, whereas, the isolates of *E. coli*, *Shigella dysenteri*ae and *Salmonella typhi* showed the less percentage 20%. All isolates of P. mirabillis were positive for serum resistance test with 100%, while the less percentage 14% observed with isolates of *Pseudomonas aeruginosa*. The results of biofilm formation test revealed that all bacterial isolates were perceived negative results except in isolates of *E. coli*, *Proteus mirabillis* and *Enterococcus faecalis*.

The genotype study investigated for three virulence genes and four antibiotics resistance genes. The results of virulence gene indicated that biofilm gene was the

most prevalence gene, where appeared in 95% of bacterial isolates followed by Iss gene 86.66% and FimH gene 85%.

bla$_{AmpC}$ and *bla*$_{TEM}$ were significantly prevalence in all bacterial isolates compare with *bla*$_{SHV\text{-}5}$ and *bla*$_{CTX\text{-}M}$ at P≤ 0.05. the percentage of the prevalence of *bla*$_{AmpC}$ and *bla*$_{TEM}$ were 93.33 and 71.66, respectively, while the less percentages were observed in *bla*$_{SHV\text{-}5}$ 20% and *bla*$_{CTX\text{-}M}$ 46.66%.

The current study showed a strong significant correlation between virulence and antibiotic resistance profile, where the statistical analysis revealed that the Person correlation was 0.957 between virulence and antibiotics determinants.

The present study demonstrated a positive relationship the ability of bacterial isolates to possess antibiotics resistance and the prevalence of virulence genes. The establishment of this correlations between resistance and virulence profiles could provide valuable input about the clinical evolution and recurrence rates of different bacterial infection.

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Chapter One

Introduction

1-1- Introduction:

There are two mechanisms play an important role in determining the outcome of a bacterial infection: the virulence of the strain and its resistance to antimicrobial agents [1].

Nowadays, a high number of treatments against infections caused by pathogens remain non-effective. It had been reported that acquisition of antimicrobial resistance may be related with the virulence in both Gram-negative and Gram-positive bacteria. Thus, acquisition of resistance promotes an increase or decrease of virulence. In addition, virulence and resistance might be contained in the same mobile genetic element and transferred together to microorganisms belonging to the same or to different species [1].

First, it is important to know what virulence means. Bacterial pathogenicity is defined as all the biochemical mechanisms that allow the microorganisms to cause infection [2]. Pathogenicity is a complex characteristic which depends on a large number of factors [3], some related to the host and its immunity, and others related to the microorganism: infectious doses, virulence factors (adhesins, invasins, toxins, etc.), and resistance to the host defense mechanisms. The word virulence is used to indicate the level of pathogenicity between different strains belonging to the same species, and is defined as the cell dose needed to cause a pathological response in the host. Thus, strains with different levels of virulence can exist within the same species. Some of these virulence factors may be contained in chromosomal regions named pathogenicity islands (PAIs) which present several common characteristics. Some of these PAIs are unstable [4]. PAIs have been defined in *Salmonella* strains, uropathogenic *Escherichia coli* strains, *Yersinia spp*., *Staphylococcus spp*., and so on [4]. Other virulence factors are found in plasmids which have the capacity to transfer between strains belonging to the

same or different species, favoring spread of virulence [5,6]. Currently, a large number of antimicrobial treatments are unsuccessful due to the increase in microorganisms' resistant to the antimicrobial agents. Acquisition of resistance may be associated with phenotypic changes in bacteria [7,8].

Virulence and resistance are similar in that most of the determinants have been transmitted between bacteria by horizontal gene transfer and the transfer of DNA is probably the most important mechanism for dissemination and co-selection of virulence and resistance properties [9].

1.2. The aim of the study

Because of:

- The Pathogenicity of bacterial strains is due to the presence of many virulence genes, located on chromosomes or plasmids or both that encodes important virulence factors, if present on the chromosome, these genes are typically found in specific regions called pathogenicity islands.
- Several strains of pathogenic bacteria have plasmids that code for antimicrobial enzymes, which promote resistance to third generation cephalosporins, β-lactam antibiotics and other antimicrobials and are associated with the difficult of treatments and high morbidity and mortality rates.
- The antimicrobial resistance often is considered a virulence trait in most pathogenic bacteria, increasing rates of antimicrobial resistance of them and the presence of putative virulence determinants and linkage of the two traits could possibly predict overall increasing virulence in these pathogen, therefor, the present study was aimed to

Investigate the presence of some virulence and antibiotic genes and to evaluate a relationship between antibiotic resistance and virulence in pathogenic bacteria from different sources.

Chapter Two

Literatures Review

2.1. Clinical importance of Pathogenic bacteria

2.1.1. *Escherichia coli*

E. coli is the head of a large bacterial family, Enterobacteriaceae or so called the enteric group; this bacterium was first discovered in human colon in 1885 by a German bacteriologist Escherich it was first called *Bacillus coli* [10].

E. coli is facultative anaerobic Gram–negative, non-spore forming rod, motile by peritrichous flagella, rare of them slightly capsulated [11], most strains of *E. coli* can grow on simple laboratory media containing glucose as the main carbon source. Most strain recovered in the clinical laboratory ferment lactose and thus grows smooth, glossy, pink colonies on MacConkey agar [12].

E. coli is an enteropathogens have many virulence factors including different toxins, enzymes and colonization factors. Specific serotypes with particular virulence factors often preferentially in fact specific extra intestinal sites. Other virulence factors including soluble and cell bound hemolysins, capsules, protease and adherence pili [13].

E. coli contains many antigens, these are O, H and K antigens, according to these antigens over 700 antigenic types (serotypes) are characterized, serotyping is still important in distinguishing the small number of strains that actually cause disease [13].

E. coli responsible about many infections in humans, it is main causative agent for, Urinary tract infections (UTI); they may cause neonatal meningitis and intestinal diseases (gastroenteritis) [10].

Five classes (virotypes) of *E. coli* that causes diarrhea diseases are now recognized enterotoxigenic *E. coli* (ETEC) enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEc). Each class falls within a serological subgroup and manifests distinct features in pathogenesis. *E. coli* produces many types of toxins such as enterotoxins including heat libel(LT), heat stable(ST), verotoxin and shiga- like toxins [10] [12] [13].

2.1.2. *Klebsiella pneumoniae*

Klebsiella genus belongs to ***Enterobacteriaceae*** family, and is composed by gram negative rod-shaped bacteria, generally encapsulated, lysine decarboxylase but not ornithine decarboxylase producers, and commonly positive in the Voges-Proskauer test [14].

K. pneumoniae is the most relevant pathogen within genus ***Klebsiella***, being responsible for 75% to 86% of ***Klebsiella*** spp infections. Additionally, ***K. oxytoca is*** the second most prevalent ***Klebsiella*** species, being responsible for 13% to 25% of infections [15].

Klebsiella spp., as human pathogens, may be responsible for both community and nosocomial infections. In the community set, ***K. pneumoniae*** is associated to several infections. It is responsible for Friedlanders pneumoniae, a community acquired pulmonary infection, which affects chronic alcoholics, and whose incidence has been decreasing over the years [14]. Additionally, three new severe clinical syndromes have been emerging in Asia. First of all, community-acquired primary pyogenic liver abscess (PLA), a ***K. pneumoniae*** infection that as emerged in the past 20 years and may or may not be associated to septic metastatic complications [16]. The majorities of PLA cases are generally associated with K1 serotype and have been detected mainly in Taiwan [17].

Considering nosocomial infections, ***Klebsiella***e are considered important opportunistic pathogenic agents, being responsible for infections mainly located in the urinary and respiratory tracts, but which may also affect soft tissues and wounds and cause septicemia [14]. Several host characteristics such as diabetes mellitus, extremes of age, renal, cardiac or pulmonary chronic diseases, as well as oncologic problems, may predispose to ***Klebsiella*** spp. infections [18]

2.1.3. *Proteus mirabillis*

Proteus belongs to the Enterobacteriaceae family within the Proteobacteria. They are Gram-negative rods with peritrichous flagella, polymorphic, with a diameter ranging between 0.4 – 0.8 μm, and characterized by rapid motility and by production of the urease enzyme. The genus *Proteus* currently consists of five named species (*P. mirabilis, P. penneri, P. vulgaris, P. myxofaciens* and *P. hauseri*) and three unnamed genomospecies (*Proteus* genomospecies 4, 5, and 6). *Proteus* from Homer's Odyssey was pursued by mortals and gods alike for his ability to foretell the future, but he evaded pursuers by taking the shape of animals, plants, water, or even fire. The term *Proteus* therefore refers to readily changing appearance: "and has the gift of endless transformation" [19].

P. mirabilis has been implicated in bacteremia, neonatal meningoencephalitis, empyema and otitis media [20]. *P. penneri* has been implicated in a case of bacteremia and concomitant subcutaneous thigh abscess in a neutropenic patient with acute lymphocytic leukemia and in nosocomial urosepsis in a diabetic patient from whom the organism was also subsequently isolated from bronchoalveolar lavage fluid and a pulmonary artery catheter tip [21]. The urease enzyme of *P. penneri* is also believed to be a leading cause of kidney stone formation; indeed, the organism has been isolated from the center of a stone removed from a patient with persistent *P. penneri* bacteriuria. *P. penneri* has also been isolated from stool and infected conjunctiva [20,21].

Nosocomial transmission has been reported in 1983 on five patients in a cardiac surgery unit with septicemia caused by either *P. mirabilis*, *Morganella morganii*, or both organisms, while other reports have included an outbreak of neonatal meningoencephalitis, infections in a hospital newborn nursery traced to a single nurse, and an outbreak in a surgical intensive care unit spread either by autoinfection or by gastrointestinal colonization prior to cross-infection [20].

2.1.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a non-fermentative, aerobic Gram negative rod, measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum. It normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, from water and soil to plant and animal tissues. Typical biochemical features of *P. aeruginosa* isolates are: positive oxidase test, growth at 42 °C, hydrolysis of arginine and gelatine, and nitrate reduction. P. aeruginosa strains produce two types of soluble pigments, pyoverdin and pyocyanin. The latter blue pigment is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is characteristic for suppurative infections caused by *P. aeruginosa* [22].

Pseudomonas aeruginosa is a gram-negative rod bacterium, which is reported to be ubiquitous in humans, animals, and the natural environment. The widespread habitat of *P. aeruginosa* makes it very difficult to control the organism in a hospital setting. Prevention of contamination is practically impossible. The main danger is the infection of patients who are immunologically compromised or those in burn units, neonatal units, and cancer wards. *P. aeruginosa* is difficult to eradicate due to a number of factors, the most important of which is the relatively poor efficacy of antibiotics against *P. aeruginosa* due to multiple resistance mechanisms expressed by the bacterium [23].

2.1.5. *Shigella dysenteriae*

Shigella species, members of the family Enterobacteriaceae, are responsible for causing acute gastroenteritis which is one of the most common causes of morbidity and mortality in children in developing countries [24]. Shigellosis is ubiquitous in impoverished populations of Asian and African countries and antibiotic-resistant strains of different Shigella species and serotypes have emerged all over the world [25].

Shigella dysenteriae is a species of the rod shaped bacterial genus Shigella. Shigella species can cause shigellosis (bacillary dysentery). Shigella are Gram negative, non-spore-forming, facultatively anaerobic, non-motile bacteria. *S. dysenteriae*, spread by contaminated water and food, causes the most severe dysentery because of its potent and deadly Shiga toxin, but other species may also be dysentery agents [24,25].

2.1.6. *Salmonella typhi*

Salmonella is a genus of the family Enterobacteriaceae and comprises a large and closely related population of medically important pathogens. It has long been associated with a wide spectrum of infectious diseases, including typhoid fever and non-typhoid salmonellosis, which cause public health problems worldwide [26].

Salmonella causes approximately 1.4 million human infections each year in the United States, resulting in 116,000 hospitalizations and 600 deaths. Most Salmonella infection is limited to uncomplicated gastroenteritis that seldom requires antimicrobial treatment. However, severe sequelae, such as bacteremia or meningitis, may develop in an approximately 5-10% of individuals infected with non-typhoid Salmonella. Invasive Salmonella infections can be fatal and antimicrobial treatment is essential in these circumstances. All Salmonella can cause extra-intestinal infections, but *S. typhi, S. paratyphi, S. choleraesuis and S. dublin* are the major serotypes which cause invasive salmonellosis in humans [26]

2.1.7. *Enterococcus faecalis*

Enterococci bacteria are facultative anaerobic gram-positive cocci, which are considered part of the normal flora in humans and animals. However, these microorganisms may be the cause of several serious systematic infections too. The two most common *Enterococcus* species, *E. faecalis* and *E. faecium* are responsible for 80-90% and 5-10% of human *enterococcal* infections, respectively. *E. faecalis* is the most common isolate of nosocomial infections, but newly, due to increasing resistance to some antimicrobial agents, especially vancomycin, *E. faecium* isolates are also being considered.4 It has been shown that separate lineages of *E. faecalis* and *E. faecium* are leading causes of the large number of the multidrug-resistant *enterococcal* infections [27].

2.1.8. *Serratia marcescens*

Serratia marcescens is a gram negative, motile, non-lactose fermenting bacillus of the family Enterobacteriaceae. Both pigmented and non-pigmented varieties exist. It can be found in soil and water and was originally considered to be only a saprophyte. It is now recognized as a formidable nosocomial pathogen1 capable of causing serious infections and often death in hospitalized patients.

The organism usually attacks compromised hosts: patients with defective immune responses or some underlying severe disability to predispose them to infection or provide an artificial portal of entry. Neonates, the immunosuppressed, patients with carcinomatosis, leukaemia or a reticulosis and those with chronic neurological and urological disorders are at risk. Prior corticosteroid therapy, the postoperative status, mechanical respiratory manipulation, instrumentation of the genito-urinary tract and multiple and "broad-spectrum" antibiotic therapy may also predispose to serratia infection [28].

2.1.9. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, round-shaped bacterium that is a member of the Firmicutes (strong cell wall), and it is a member of the normal flora of the body, frequently found in the nose, respiratory tract, and on the skin [29].

S. aureus is a facultative anaerobic, Gram-positive coccal (round) bacterium also known as "golden staph" *S. aureus* is non-motile and does not form spores [30].

S. aureus has the ability to cause skin infection, food poisoning, bone and joint infections, bacteremia, medical implant infection and animal infections [30].

The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Despite much research and development, no vaccine for *S. aureus* has been approved [29,30].

2.1.10. *Streptococcus pyogenes*

Streptococcus pyogenes belongs to the serological group A among the streptococci (group A Streptococcus, GAS) and is an exclusively human pathogen. GAS causes significant disease worldwide and adds a large burden to national health care systems. An excellent compilation of data and estimates of the global burden of GAS diseases from 2005 revealed 616 million cases of pharyngitis, 111 million cases of pyoderma and at least 517,000 deaths due to severe invasive diseases and sequelae. This dataset is manifesting the important status of GAS among bacterial pathogens and is an impressive documentation of GAS impact on global mortality and morbidity.

The virulence potential of this species is tremendous. Interactions with humans range from asymptomatic carriage over mild and superficial infections of skin and mucosal membranes up to systemic purulent toxic-invasive disease manifestations. This places GAS among the most important Gram-positive bacterial pathogens [31].

2.2. Antimicrobial resistance

Bacteria can present different resistance phenotypes, in which multidrug resistant is defined if bacteria present resistance to at least one agent in three or more antimicrobial categories [32]. Thus, considering the progressive risk of occurrence of untreatable infections due to multidrug resistant bacteria, antimicrobial resistance has become a central problem worldwide.

Multidrug resistance in Gram-negative bacteria, particularly ***Enterobacteriaceae*** such as ***E. coli*** and ***K. pneumoniae***, has become one of the biggest global concerns. Infections with these bacteria lead to prolonged hospital admissions and higher mortality rates [33].

In general, resistance mechanisms are mainly due to increased efflux of the antibiotic, decreased wall penetration, target modification, or enzymatic alteration or inactivation of the antimicrobial agent (Figure 2.1) [32].

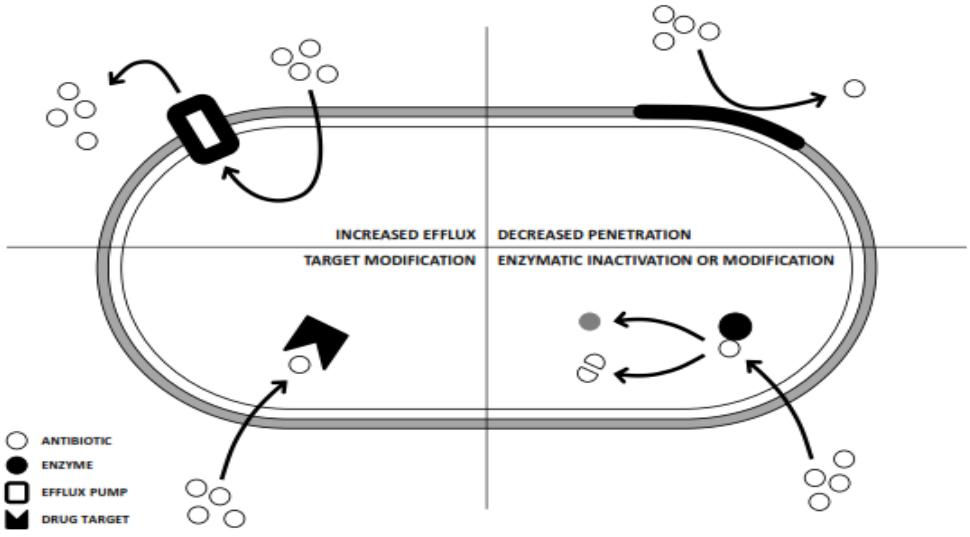


Figure 2.1: General mechanisms of antimicrobial resistance.

2.2.1. Resistance to beta-lactams

Resistance to β-lactams may be a result of several mechanisms including modification of penicillin-binding proteins, loss of porins, production of β-lactamases or overexpression of efflux pumps [34]. Nonetheless, in Gram-negative bacteria, β-lactams resistance is mainly due to the hydrolytic action of β-lactamases. These enzymes inactivate beta-lactam antibiotics by hydrolyzing the peptide bond of the four-membered beta-lactam ring [35].

Four major groups of β-lactamases can be identified considering the substrate characteristics: penicillinases, AmpC-type cephalosporinases, Extended-Spectrum βlactamases (ESBLs) and carbapenemases, with ESBLs being the largest group [36].

2.2.1.1. Naturally occurring resistance

Several gram-negative bacteria have an intrinsic chromosomally mediated βlactamase [37]. *E. coli* naturally present insignificant levels of a non-inducible chromosomal *AmpC* β-lactamase. Due to its limited capacity, this *AmpC* is only responsible for resistance to agents which have a poor capacity of penetration like isoxazolyl penicillins and benzylpenicillin, and to cefsulodin. *E. coli* carrying only this non-inducible *AmpC* are characteristically susceptible to ampicillin and the narrow-spectrum cephalosporins, including cephalothin and cephalexin [38].

2.2.1.2. Extended spectrum β-lactamases

The designation ESBL was used to define β-lactamases with a broad spectrum of hydrolysis, resulting from the occurrence of amino acid substitutions in the structure of the enzymes [39].

ESBLs belong to Class A according to Ambler's classification, and group 2 be functional group of Bush's classification [40]. These β-lactamases are active against broad-spectrum cephalosporins (such as ceftazidime, cefotaxime and ceftriaxone) but are inhibited by β-lactamases inhibitors like tazobactam, sulbactam

and clavulanic acid [41]. The majority of the ESBLs detected among *Enterobacteriaceae* belong to three main families: TEM, SHV and CTX-M.

2.2.1.2.1. TEM β-lactamases

The TEM (for Temoneira patient's name) β-lactamase family is composed of more than 219 variants [42].

The first enzyme of this family described, the native TEM-1 β-lactamase is capable of hydrolyzing ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has insignificant activity against extended-spectrum cephalosporins. In addition, it is inhibited by clavulanic acid. TEM-2, was the first variant identified, but it is not considered an ESBL as the hydrolitic profile is identical to TEM-1. Besides an amino acid substitution (Q39K), TEM-2 differs from TEM-1 because it presents a more active promoter and has a different isoelectric point [42].

The TEM-type ESBLs are derivates of TEM-1 and TEM-2. Amino acid substitutions acting alone or with additional other structural gene mutations, have been detected in over than 90 described TEM-1-or TEM-2-derived enzymes. Each TEM-derived slightly differs in the hydrolytic profile, and therefore one ESBL may hydrolyze a specific extended spectrum cephalosporin in a more efficient way than another ESBL [43].

The most disseminated TEM-type ESBLs among ***Enterobacteriaceae*** in Europe in the clinical setting are TEM-24, TEM-4 and TEM-52, while in isolates from animals TEM-52, TEM-106 and TEM-116 are the most common [44].

2.2.1.2.2. SHV β-lactamases

The SHV (for Sulfhydryl Reagent Variable) β-lactamase family comprises 185 variants [38].

The native SHV-1 β-lactamase is an enzyme which may be chromosomally or plasmid encoded, and is responsible for resistance to Penicillins [38].

SHV-type ESBLs are point mutants of both narrow-spectrum β-lactamases SHV-1 or SHV-11 that had origin in the ***K. pneumoniae*** chromosome (Poirel et al., 2012). The hydrolytic spectrum of SHV-type ESBLs includes activity against oxyimino-β- lactams such as cefotaxime, ceftazidime, ceftriaxone and aztreonam [36].

There are more than 45 SHV-type ESBLs have been described so far. In Europe, SHV-5 is widespread in the clinical setting, while SHV-12 has been reported in both human and animal strains [44].

2.2.1.2.3. CTX-M β-lactamases

CTX-M-type (for Cefotaximase firstly identified in Munich) β-lactamases are a family of ESBLs which comprise 157 elements.

These β-lactamases confer resistance to penicillins and expanded-spectrum cephalosporins, and the majority of the variants present higher rates of hydrolysis to cefotaxime than to ceftazidime (Bonnet, 2004). Nonetheless, some of these enzymes, including CTX-M-15, -16, - 25, -27, -28, -29 and- 32 have an Asp240Gly substitution which enhances the catalytic activity against ceftazidime [45].

CTX-M enzymes are also susceptible to clavulanate and tazobactam βlactamase inhibitor combinations, with tazobactam presenting greater inhibitory activity compared to clavulanic acid [46].

2.3. Bacterial virulence factors

The virulence factor concept has been a powerful engine in driving research and the intellectual flow in the fields of microbial pathogenesis and infectious diseases. At a practical level the finding that effective immune responses often target virulence factors provides a roadmap for future vaccine design. However, there are significant limitations to this concept, which are rooted in the inability to define virulence and virulence factors in the absence of host factors and the host response. In fact, this concept appears to work best for bacterial pathogens,

being less well suited for viruses and commensal organisms with pathogenic potential [47].

Biofilm formation consists of initial bacterial adherence to the surface, followed by multiplication and production of extracellular polymeric matrix, which cause cell aggregation [48].

Type 1 fimbriae, which may promote bacterial adhesion and biofilm formation, recognize mannose oligosaccharides naturally presented on glycoprotein molecules of the host cell surface. Type 1 fimbriae are encoded by the *fim* gene cluster and consist of a major protein, *FimA*, associated with ancillary proteins *FimF* and *FimG* and the adhesion protein *FimH* [49].

The pap (Pyelonephritis-associated pili) gene cluster consists of 11 genes encoding the main component of the fimbria rod (*PapA*), *papEF*, which encode adaptor subunits, and a terminal adhesion *PapG* [49].

The ISS (increased serum survival) gene and its protein product (ISS) of pathogenic bacteria are important characteristics of resistance to the complement system [50].

The presence of a gene for increased serum survival, iss, is strongly correlated with *Escherichia coli* isolated from birds with colibacillosis. Therefore, the iss gene and its protein product, Iss, are potential targets for detection and control of avian colibacillosis [50].

Chapter Three

Materials and Methods

3- Materials and Methods:

3.1. Bacterial isolates

A total of 60 pathogenic bacterial isolates were obtained from PhD student at University of Al-Kufa/ College of Science, these pathogenic bacteria were isolated from different infections. These isolates encompassed 16 isolates of *E. coli*; 9 isolates of *Pseudomonas aeruginosa* and *Salmonella typhi*; 5 isolates of *Proteus mirabillis*; 4 isolates of *Klebsiella pneumonia*, *Shigella dysenteriae*, *Serratia marcescens*; 3 isolates of *Enterococcus faecalis*, *Staphylococcus aureus* and *Streptococcus pyogenes*.

3.2. Materials

The laboratory instruments and equipment, culture media, commercial kits and primers, which used in this study are illustrated in table (3-1), (3-2), (3-3), (3-4), respectively.

Table (3-1): The instruments and equipments used in this study with their remarks.

No.	Instrument	Company/ Country of Origin
1	Autoclave	Mammert/Germany
2	Benson Burner	Mammert/Germany
3	Camera	Canone/Japan
4	Centrifuge	Hettich, Germany
5	Disposable Petri dishes	Al-Hani company / Lebanon
6	Electric Oven	Mammert/Germany
7	Electrophoresis	Shando, scientific co. / UK
8	Eppendorf tubes	Sterellin Ltd. / UK
9	Hot plat stirrer	Labtech /Korea
10	Incubator	Mammert/Germany
11	Inoculating loop	Japan
12	Light Microscope	Olympus/Japan

13	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
14	Pipette	Volac, U.K
15	Plain tubes (10) and (15)	Bromed, USA
16	Refrigerator	Concord /Lebanon
17	Sensitive Balance	Sartorius/Germany
18	Standardized loop	Himedia, India
19	Sterilized cotton swabs	Sterile EO. / China
20	Thermocycler PCR	MWG Biotch /Germany
21	Vortex	CYAN/ Belgium
22	Water Bath	Mammert/Germany
23	UV gel documentation	GFL-Germany
24	ELISA microplate reader and washer	GFL-Germany

Table (3-2): Cultural media that used in this study with their remarks.

No.	Culture media	Company / origin
1	Blood Agar (BA) Base	Mumbai (India)
2	Brain Heart Infusion Broth (BHIB)	
3	MacConkey agar (MA)	
4	Nutrient Agar (NA)	
5	Nutrient Broth (NB)	
6	Tryptic soy broth	

Table (3-3): The kits used in this study with their remarks.

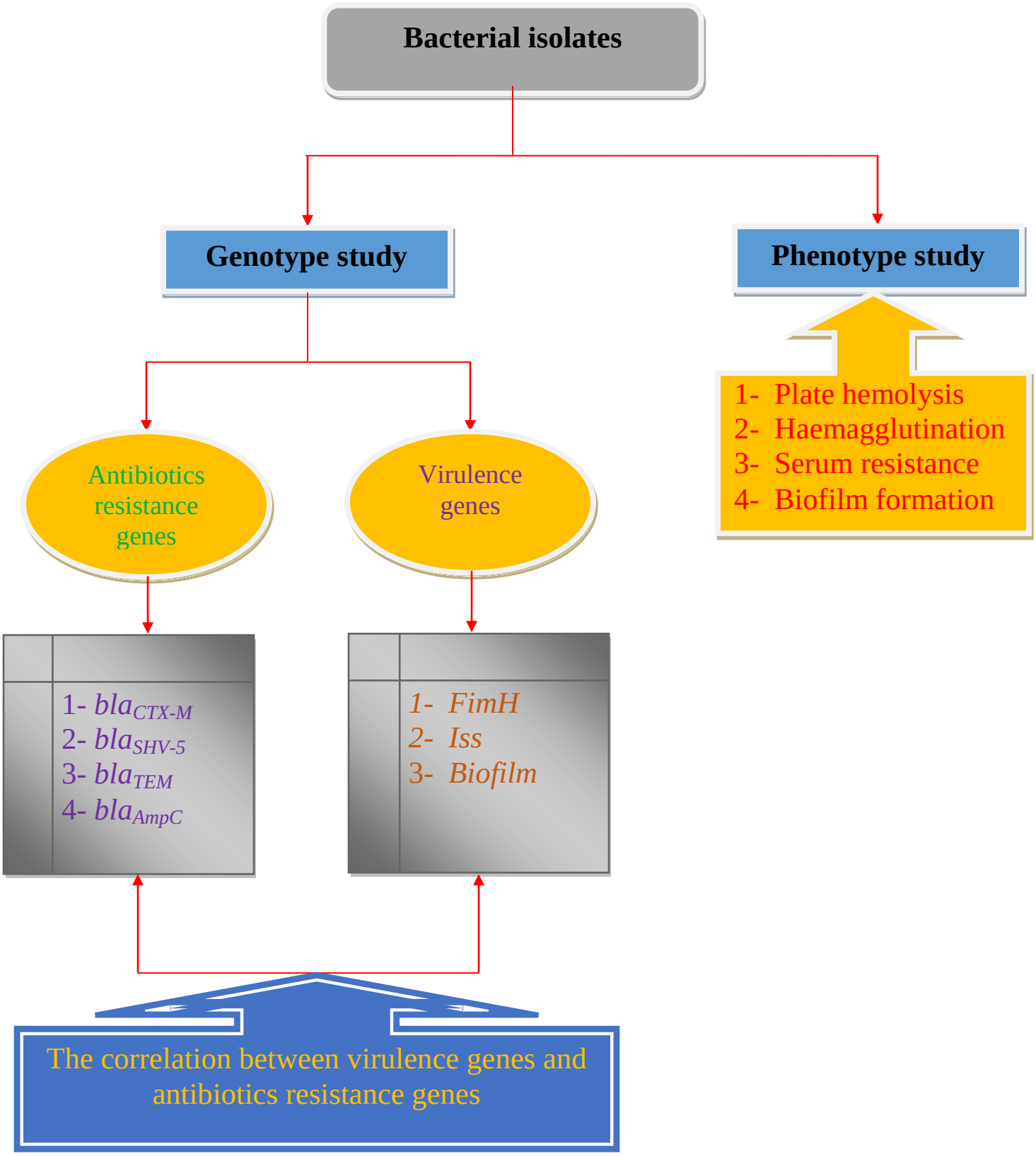
No.	Kit	Company	Country
1	Genomic DNA Extraction Kit	Geneaid	USA
2	iNtron PCR PreMix	Bioneer	South Korea

Table (3-4): The primers used in this study and their product size designer, sequencing, purpose, and Provider Company.

Target gene	Primer sequence (5'- 3')		Product size (bp)	Purpose	Company/ Country
fimH	F	TACTGCTGATGGGCTGGTC	640 bp	Detection of fimbriae H gene	Bioneer South, Korea
	R	GCCGGAGAGGTAATACCCC			
Iss	F	GGCAATGCTTATTACAGGATGTGC	260 bp	Detection of serum increase survival gene	Bioneer South, Korea
	R	GAGCAATATACCCGGGCTTCC			
Biofilm	F	GATTCAATTTTGGCGATTCCTG	225 bp	Detection of biofilm gene	Bioneer South, Korea
	R	TAATGAAGTCATTCAGACTCATCC			
$bla_{CTX\text{-}M}$	F	AGCGATAACGTGGCGATGAA	247bp	Detection of Cefotaximase gene	Bioneer South, Korea
	R	TCATCCATGTCACCAGCTGC			
$bla_{SHV\text{-}5}$	F	CCGCCATTACCATGAGCGAT	410bp	Detection of sulfhydryl variable gene	Bioneer South, Korea
	R	AATCACCACAATGCGCTCTG			
bla_{TEM}	F	GGTGCACGAGTGGGTTACAT	531bp	Detection of temoneira gene	Bioneer South, Korea
	R	TGCAACTTTATCCGCCTCCA			
bla_{AmpC}	F	AAACGACGCTCTGCACCTTA	670bp	Detection of ampicillin gene	Bioneer South, Korea
	R	TGTACTGCCTTACCTTCGCG			

3.3. Methods

The design of study explained in the figure (3-1):



3.3.1. Maintenance and storage of bacterial isolates

To store isolates for long time without losing their genetic characteristic, nutrient agar plate streaked with a single colony of the bacteria, after appearance of growth at 37˚C, 5 ml of nutrient broth and BHI broth in separated vials supplemented with sterilized 15% glycerol was inoculated with single colony. Then stored at -20˚C in addition to slants for 6-8 months and at 4 ^{o}C for month culturing on new culture media [51].

3.3.2. Phenotypic Detection of Virulence Factors:

3.3.2.1. Plate hemolysis

Isolates were tested for the production of a hemolytic phenotype on blood agar plates containing 5% (vol/vol) blood. Production of hemolysis was read after overnight incubation at 37 ^{o}C [52].

3.3.2.2. Haemagglutination

The haemagglutination was detected by clumping of erythrocytes by fimbriae of bacteria. This test was carried out as in the direct bacterial haemagglutination test – slide method as follow [53]:

1) Isolates were inoculated into 1% nutrient broth and incubated at 37°C for 48 hours for full fimbriation.
2) A panel of red blood cells was selected by obtaining blood from human (blood group 'O').
3) The red blood cells were then washed three times in normal saline and made up to a 3% suspension in fresh saline.
4) They were used immediately or within a week when stored at 3-5°C.
5) On a glass slide, one drop of the RBC suspension was added to a drop of the broth culture and slide was rocked at room temperature for 5 minutes.
6) Presence of clumping was taken as positive for haemagglutination.

3.3.2.3. Serum Resistance

Serum resistance was analyzed using a turbid metric assay as follow [54]:

1) One hundred and fifty microliters of serum were mixed with 50 µl of bacterial suspension in a 96 well microplate.
2) Each isolate was tested in duplicate and negative controls (0.9% NaCl instead of serum) were included.
3) The initial absorbance at 630 nm was measured, and compared with the absorbance after 3 hours of incubation, using a micro plate reader.
4) The final absorbance was determined as the average of the two replicates, and the percentage remaining absorbance relative to the initial absorbance was calculated.
5) If the remaining absorbance after 3 hours (OD630, 3 h) was higher than 100% (relative to the initial absorbance), isolates were designated serum resistant and less than 100% were considered sensitive.

3.3.2.4. Biofilm Formation

Microtitre-plate test was used to detect the biofilm formation for all bacterial isolates as follow [55]:

1) A 20 µl of bacterial ioslates overnight cultures were used to inoculate 96-microtitier wells plate containing 180 µl of TSB. Negative control wells contained the broth only.
2) Cultures were removed and the wells were rinsed with PBS (pH 7.2).
3) After drying at room temperature for 15 min., 200 µl per well of crystal violet (1%) was added to the wells for 20 min.
4) The stained biofilms were rinsed three times with PBS (pH 7.2), and allowed to dry at room temperature for 15 min., and then extracted twice with 200 µl per well of 95% ethanol.
5) The OD_{630} of each well was estimated using automatic microtiter plates reader. All assays were performed in duplicate.

6) The average OD values were calculated for all tested isolates and negative controls.
7) The cut-off value (ODc) was established, it was defining as a three standard deviations (SD) above the OD mean of the negative control.
8) The OD values of a tested isolates was expressed as average OD value of the isolate reduced by ODc value. OD = average OD of isolate – ODc).
9) Finally, the biofilm results of isolates were divided into the following categories:
    - Non-biofilm producer (OD ≤ ODc)
    - Weak biofilm producer (ODc < OD ≤ 2 x ODc)
    - Moderate biofilm producer (2 x ODc < OD ≤ 4 x ODc)
    - Strong biofilm producer (4 x ODc < OD)

3.3.3. Genotypic detection of virulence genes

3.3.3.1. Preparation bacterial isolates for genomic DNA extraction

All bacterial isolates were subcultured on nutrient agar and incubated for 18-24 hours at 37 ^{o}C. 5 ml of nutrient broth in 60 sterile screwcaps (5 ml for each screwcaps) was prepared and autoclaved for 15 min, after that inoculated by single colony of each bacterial isolates previously prepared and then incubated for 18-24 hours at 37 ^{o}C [51].

3.3.3.2. Genomic DNA extraction

Genomic DNA of bacterial isolates was extracted by using Genomic DNA Mini Kit, as follows:

1) One ml of (18 hours) incubated cultured bacterial cells was transferred to a 1.5 ml micro-centrifuge tube then centrifuged in high-speed centrifuge at 15000 rpm for one minute, then the supernatant was discarded.
2) Lysozyme buffer (200 µl) was added to the tube and the cell pellet was re-suspended by shaking vigorously by vortex, incubated at room temperature for 10 minutes, and the tubes inverted every 3 minutes through incubation periods.

3) GB buffer (200 µl) were added to each tube and mixed by shaking vigorously for 5 seconds. Then the tubes were incubated at 60ºC for 10 minutes and inverted every 3 minutes through incubation periods.
4) Absolute ethanol (200 µl) were added to the clear lysate and immediately mixed by shaking vigorously, then precipitated broke it up by pipetting.
5) A GD column was placed in a 2 ml collection tube and all the mixture was transferred (including any precipitate) to the GD column. Then, it was centrifuged at 15,000 rpm for 2 minutes. The 2 ml collection tubes containing the flow-through were discarded and placed the GD column was placed in a new 2 ml collection tube.
6) W1 buffer (400µl) were added to the GD column, then centrifuge at 15,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.
7) Wash Buffer (ethanol) 600µl were added to the GD column. Then, it was centrifuged at 15,000 rpm for 30 seconds. The flow-through was discarded and the GD column was placed back in the 2 ml collection tube.
8) All the tubes were centrifuged again for 3 minutes at 15,000 rpm to dry the column matrix.
9) The dried GD column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 µl of pre-heated elution buffer was added to the center of the column matrix.
10) The tubes were left to stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then, it was centrifuged at 15,000 rpm for 30 seconds to elute the purified DNA.

3.3.3.3. Genomic DNA electrophoresis:

The extracted genomic DNA was visualized by red safe staining after gel electrophoresis as follow [56]:

1) 1.3% Agarose gel was prepared by dissolving 1.3 g of agarose with 100 ml of 1X TBE, after that, left to cool 50°C.
2) Then 5µ of red safe stain were added into agarose gel solution.
3) Agarose gel solution was poured in tray then, the comb was fixed in proper position, after that, it was left to solidify for 15 minutes at room temperature. Then, the comb was removed gently from the tray.
4) The gel tray was fixed in electrophoresis chamber and filled by 1X TBE buffer.
5) 10µl of extracted genomic DNA was added into each comb well and 10ul of (100bp Ladder) in one well.
6) Then, the electric current was applied at 100 volts for 45 min.
7) Finally, the bands of genomic DNA were visualized by using gel documentary system.

3.3.3.4. Preparation of PCR master mix:

Polymerase chain reaction master mix reaction for all virulence genes was prepared by using iNtron Kit and this master mix was done according to company instructions as follows**:**

PCR Master mix reaction components		**Volume**
DNA template		2 µl
Primers	F. primer	1 µl
	R. primer	1 µl
PCR water		16 µl
Total volume		20 µl

3.3.3.5. PCR Thermoscycler Conditions:

PCR thermocycler conditions for all virulence genes were done by using conventional PCR thermocycler system as follows:

Biofilm gene			
PCR cycle	**repeat**	**Temp.**	**Time**
Initial denaturation	1	94C	2 min
Denaturation	35	94C	40 sec.
Annealing		48C	1 min
Extension		72C	1 min
Final extension	1	72C	5 min
Hold	-	4C	-
***FimH* and *Iss* genes**			
PCR cycle	**repeat**	**Temp.**	**Time**
Initial denaturation	1	94C	2 min
Denaturation	40	94C	40 sec.
Annealing		50C	1 min
Extension		72C	1 min
Final extension	1	72C	5 min
Hold	-	4C	-

3.3.3.6. Gel electrophoresis

PCR products of All virulence genes were analyzed by loading in 1.3% Agarose as follows [56]:

1) 1.3% Agarose gel was prepared by dissolving 1.3 g of agarose with 100 ml of 1X TBE, after that, left to cool 50°C.
2) Then 5µ of red safe stain were added into agarose gel solution.
3) Agarose gel solution was poured in tray then, the comb was fixed in proper position, after that, it was left to solidify for 15 minutes at room temperature. Then, the comb was removed gently from the tray.

4) The gel tray was fixed in electrophoresis chamber and filled by 1X TBE buffer.
5) 10µl of PCR product was added into each comb well and 10ul of (100 bp Ladder) in one well.

a) Then, the electric current was applied at 100 volts for 45 min.

b) PCR products 225 bp, 260 bp and 640 bp as specific for biofilm gene, *iss* gene and *FimH* gene, respectively were visualized by using gel documentary system.

3.3.4. Genotypic detection of antibiotics resistance genes

3.3.4.1. Preparation of PCR master mix:

The PCR master mix was prepared as described in (3.3.3.4).

3.3.4.2. PCR Thermoscycler Conditions

PCR thermocycler conditions for all antibiotics resistance genes were done by using conventional PCR thermocycler system as follows:

bla$_{CTX-M}$, bla$_{SHV-5}$, bla$_{TEM}$ and bla$_{AmpC}$			
PCR cycle	**repeat**	**Temp.**	**Time**
Initial denaturation	1	94C	5 min
Denaturation	30	95C	30 sec.
Annealing		58C	30 sec.
Extension		72C	1 min
Final extension	1	72C	5 min
Hold	-	4C	-

3.3.4.3. Gel electrophoresis

PCR products of All antibiotics resistance genes were analyzed by loading in 1.3% Agarose as described in (3.3.3.6) [56] and PCR products 247 bp, 410 bp, 531 bp and 670 bp as specific for *bla$_{CTX-M}$, bla$_{SHV-5}$, bla$_{TEM}$ and bla$_{AmpC}$*, respectively were visualized by using gel documentary system.

3.3.5. Statistical Analysis:

Statistical analysis was performed using statistical software package (IBM, SPSS V.20). Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages).

Chapter Four

Results and Discussion

4. Results and discussion

4.1. Results

4.1.1. Phenotype results

4.1.1.1. Plate Hemolysis

The present study has been identified that some bacterial isolates were produced hemolysin. The results revealed that 80% of *E. coli*,100% *Klebsiella pneumoniae*, 60% *Salmonella typhi*, 100% *Serratia marcescens*, 40% *Shigella dysenteriae*, 40% *Pseudomonas aeruginosa* were positive for the production of hemolysin, while all *Proteus mirabilis* isolates were negative for hemolysin production as shown in the figure (4.1).

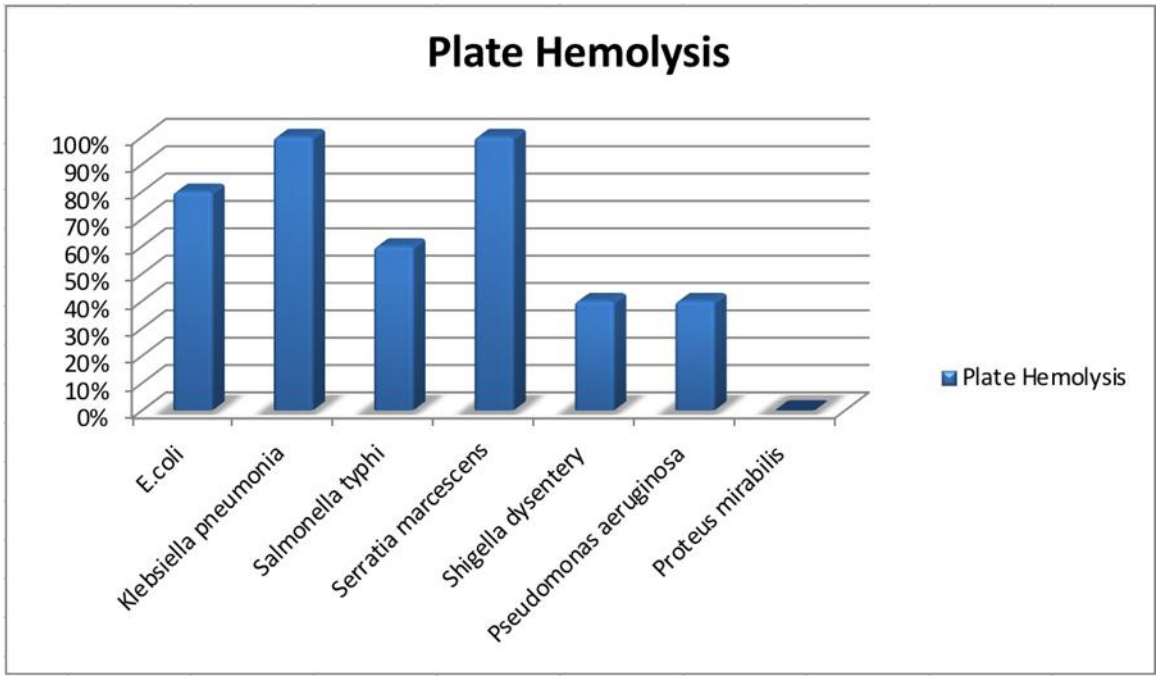


Figure (4.1): The percentages of plate hemolysis test of bacterial isolates.

4.1.1.2. Haemagglutination test

The current study conducted a slide hemolysis method for bacterial isolates and the results showed the following positive percentages: *E. coli*, *Shigella dysenteri*ae and *Salmonella typhi* 20%, *Klebsiella pneumonia* 80%, *Pseudomonas aeruginosa* 60%, *Proteus mirabilis* 50% and *Serratia marcescens* 100% as shown in the figure (4.2).

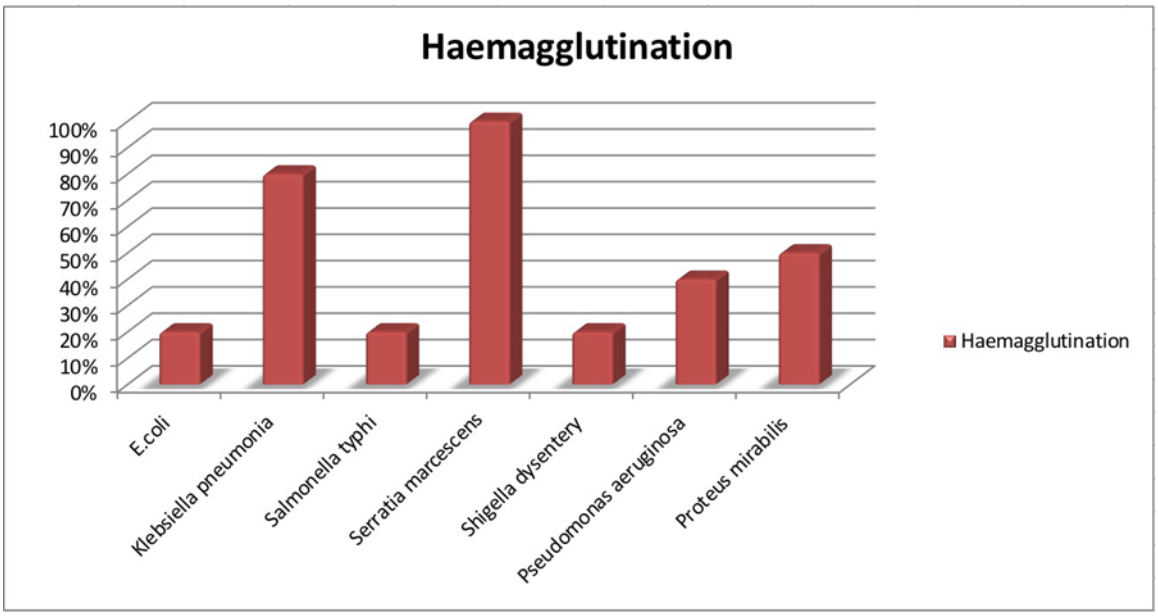


Figure (4.2): The percentages of haemagglutination test of bacterial isolates.

4.1.1.3. Serum Resistance

The bacterial isolates were also tested for serum resistance at an absorbance of 630 nm. The results showed that 100% of *Proteus mirabilis* isolates and 57% of *E. coli* isolates were positive, while 50% of *Shigella dysenteriae*, *Streptococcus pyogenes* and *Enterococcus faecalis* isolates were positive. The other positive percentages were 33%, 25%, 20%, 17% and 14% for *Staphylococcus aureus*, *Serratia marcescens*, *Klebsiella pneumonia*, *Salmonella typhi* and *Pseudomonas aeruginosa*, respectively as shown in the figure (4.3).

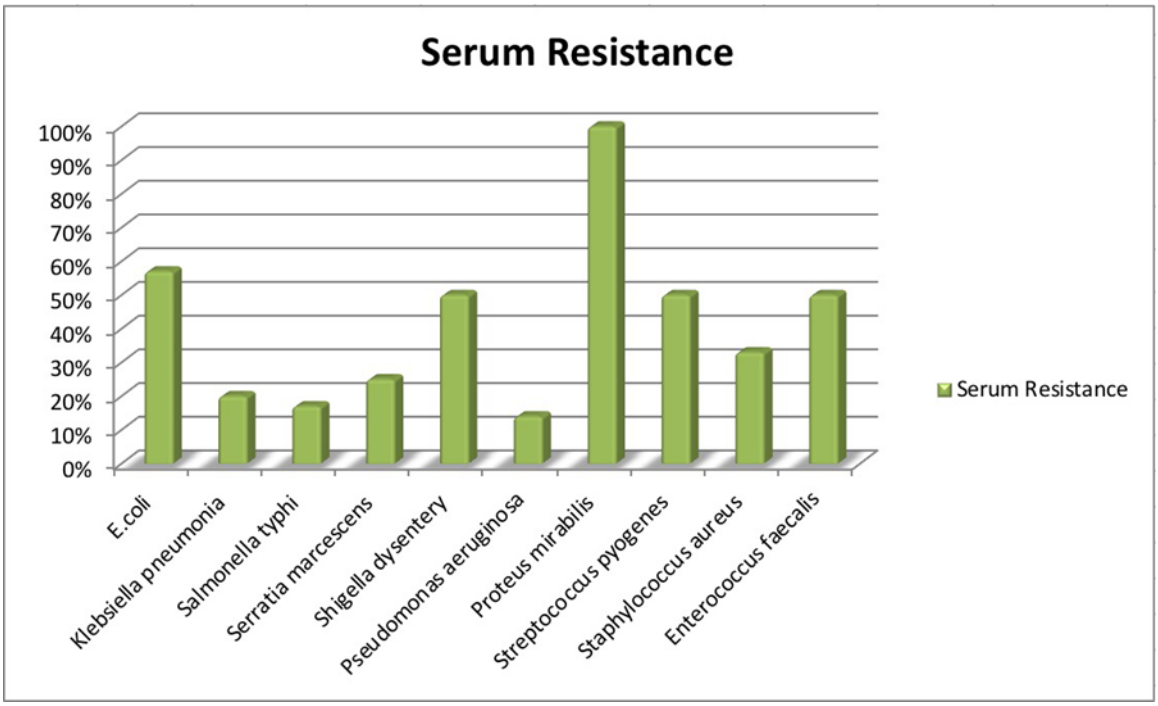


Figure (4.3): The percentages of serum resistance test of bacterial isolates.

4.1.1.4. Biofilm Formation

The results of biofilm formation test classified the bacterial isolates into three categories, strong biofilm, weak biofilm and negative biofilm. Strong biofilm was observed in 83.33% of *E. coli* isolates; 50% of *Shigella dysenteriae*, *Streptococcus pyogenes* and *Enterococcus faecalis*; 40% of *Klebsiella pneumonia*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The weak biofilm results were observed in 100% of *Proteus mirabilis*; 60% of *Salmonella typhi*; 50% of *Serratia marcescens* and *Enterococcus faecalis;* 40% of *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, while 37.5%, 25% and 16.67% were detected in *Shigella dysentery*, *Streptococcus pyogenes* and *E. coli*. The negative biofilm results were perceived in all bacterial isolates except in *E. coli*, *Proteus mirabilis, Salmonella typhi* and *Enterococcus faecalis* as shown in the figure (4.4)

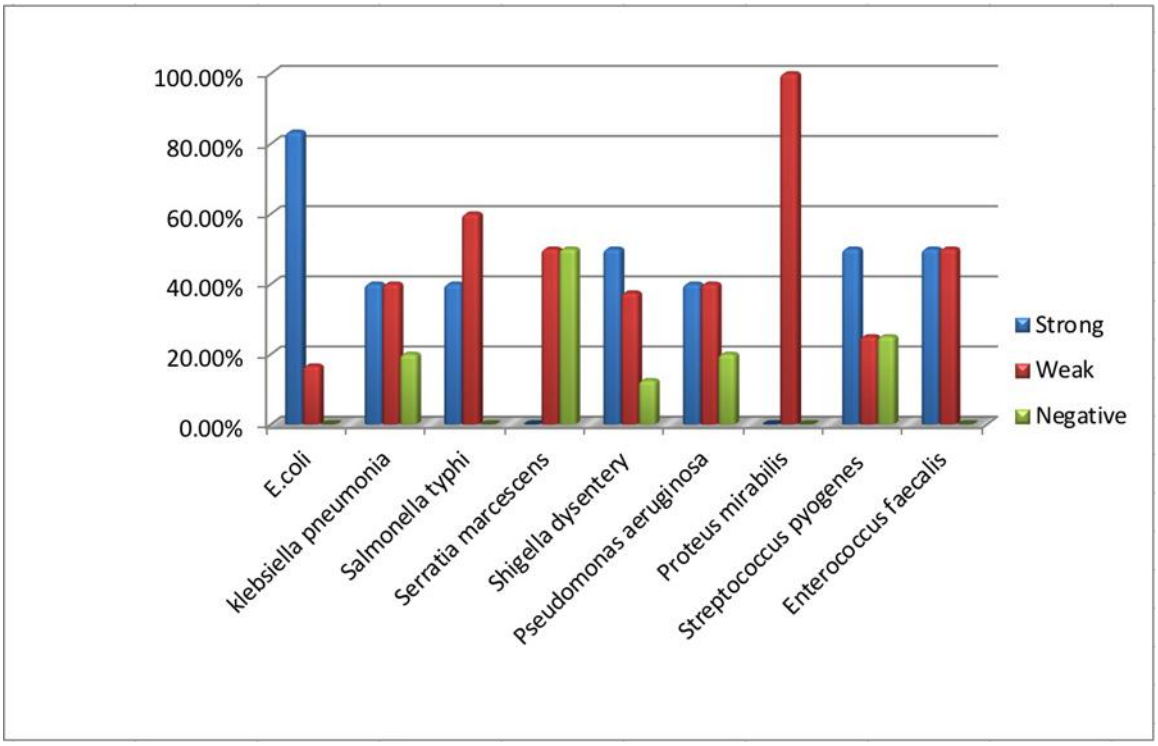


Figure (4.4): The percentage of biofilm formation test of bacterial isolates.

4.1.2. Genotype results

4.1.2.1. Virulence genes

4.1.2.1.1. Biofilm gene

The results of biofilm detection by PCR technique revealed that all isolates of *Proteus mirabilis*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Shigella dysenteriae*, *Salmonella typhi*, *Serratia marcescens*, *Staphylococcus aureus and Streptococcus pyogenes* were positive in 100%, while 93.75% and 77.77% of *E. coli* and *Pseudomonas aeruginosa*, respectively were positive for the gene of interest table (4.1). All bacterial isolates were yielded the same band size (225 bp) which was the product size of primers used for identification as shown in the figure (4.5) .

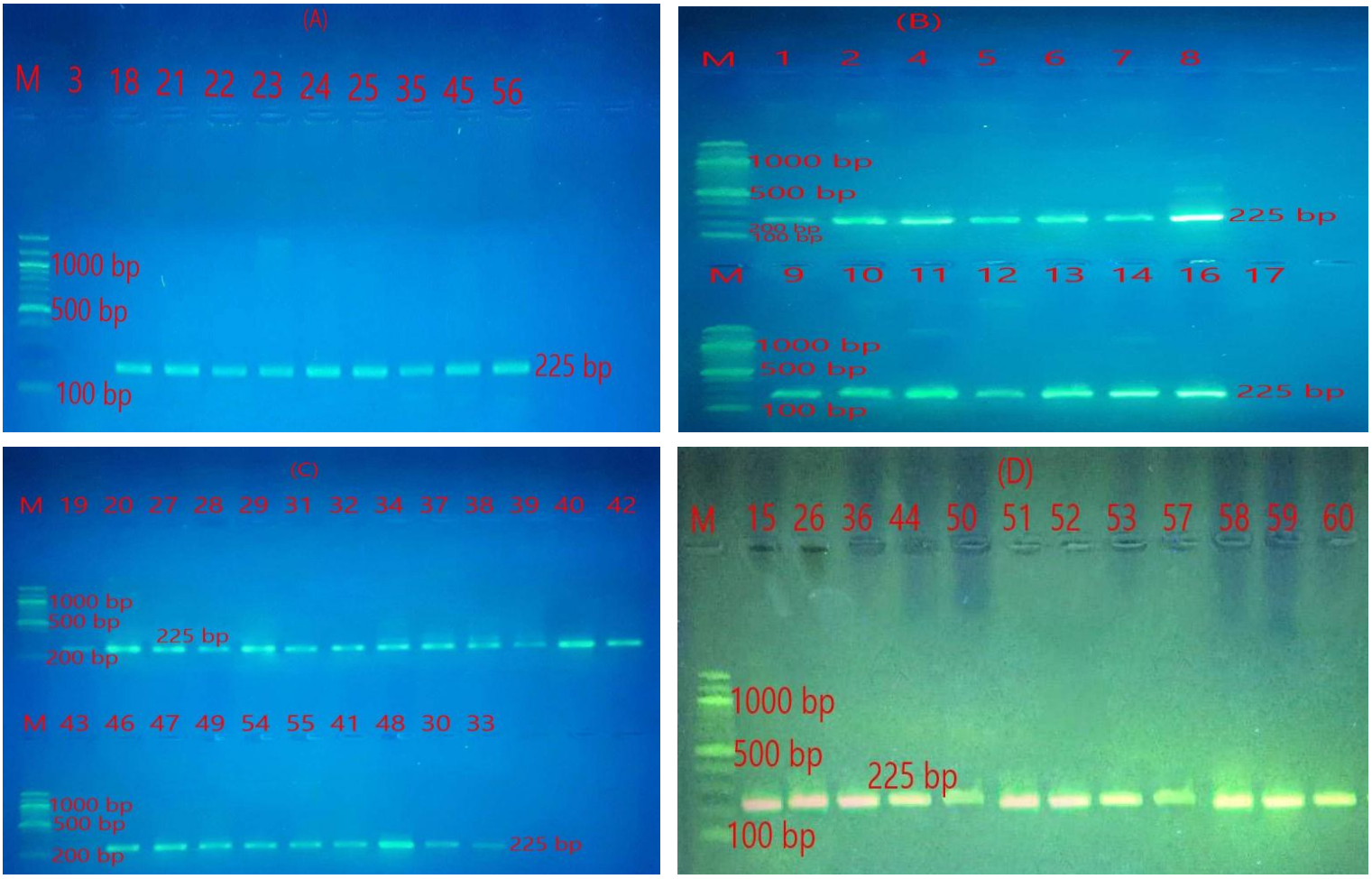


Figure (4.5): Products of polymerase chain reaction performed with biofilm gene of bacterial isolated. M: 100 bp DNA ladder. *E. coli* (5, 6, 13, 15, 16, 18, 19, 24, 25, 33, 36, 42, 43, 44, 51, 53); *P. aeruginosa* (3, 17, 26, 29, 31, 35, 39, 40, 58); *S. typhi* (2, 4, 8, 21, 27, 28, 34, 38, 41); *Sh. Dysenteriae* (12, 20, 22, 48); *K. pneumoniae* (9, 23, 32, 49); *S. aureus* (10, 11, 45), *E. faecalis* (47, 55, 56); *S. pyogenes* (1, 30, 37); *S. marcescens* (7, 14, 46, 50); *P. mirabillis* (52, 54, 57, 59, 60).

4.1.2.1.2. *FimH* gene

The present study showed that *FimH* gene was detected in all bacterial isolates of *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Serratia marcescens* and *Streptococcus pyogenes* with 100%. The other percentages of *FimH* gene were 87.5%, 75%, 66.66% and 55.55 for *E. coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Salmonella typhi*,respectively table (4.1), where all bacterial isolates gave the same band size of the interested gene (640 bp) as shown in the figure (4.6).

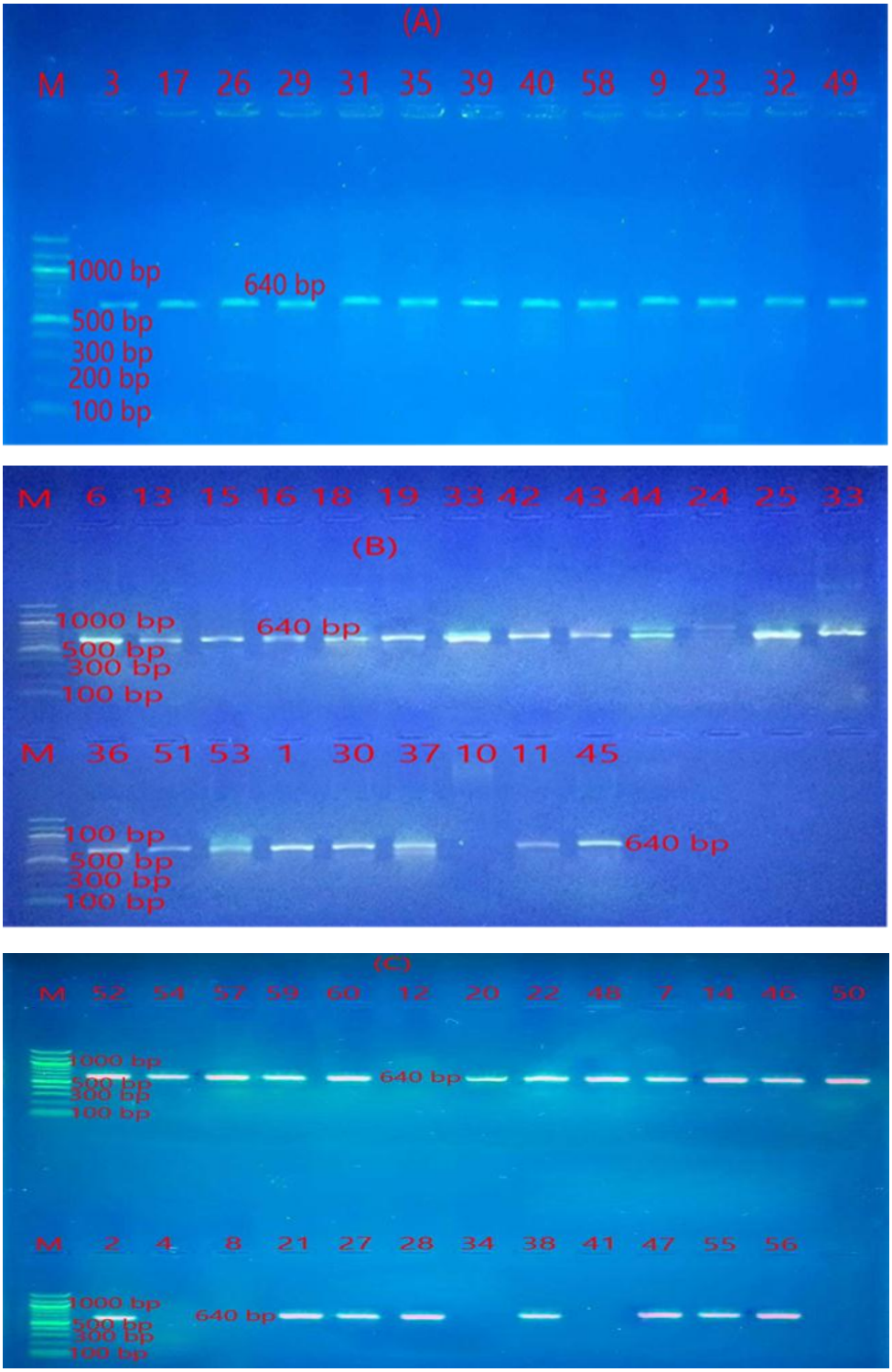


Figure (4.6): Products of polymerase chain reaction performed with *FimH* gene of bacterial isolated. M: 100 bp DNA ladder. (A): *P. aeruginosa* (3, 17, 26, 29, 31, 35, 39, 40, 58)*; K. pneumoniae* (9, 23, 32, 49). (B): *E. coli* (5, 6, 13, 15, 16, 18, 19, 24, 25, 33, 36, 42, 43, 44, 51, 53); *S. pyogenes* (1, 30, 37); *S. aureus* (10, 11, 45). (C): *P. mirabillis* (52, 54, 57, 59, 60)*; Sh. Dysenteriae* (12, 20, 22, 48)*; S. marcescens* (7, 14, 46, 50)*; S. typhi* (2, 4, 8, 21, 27, 28, 34, 38, 41); *E. faecalis* (47, 55, 56).

4.1.2.1.3. *Iss* gene

The current study of increase serum survival (*Iss*) gene by PCR explained that the gene of interest was observed with 100% in all bacterial isolates of *Proteus mirabilis*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella dysenteriae* and *Streptococcus pyogenes*. Also, 81.25% of *E. coli*, 66.66% of *Staphylococcus aureus* and 55.55% of *Salmonella typhi* indicated the presence of *Iss* gene table (4.1), where all these isolates provided the same band size (260 bp) as shown in the figure (4.7).

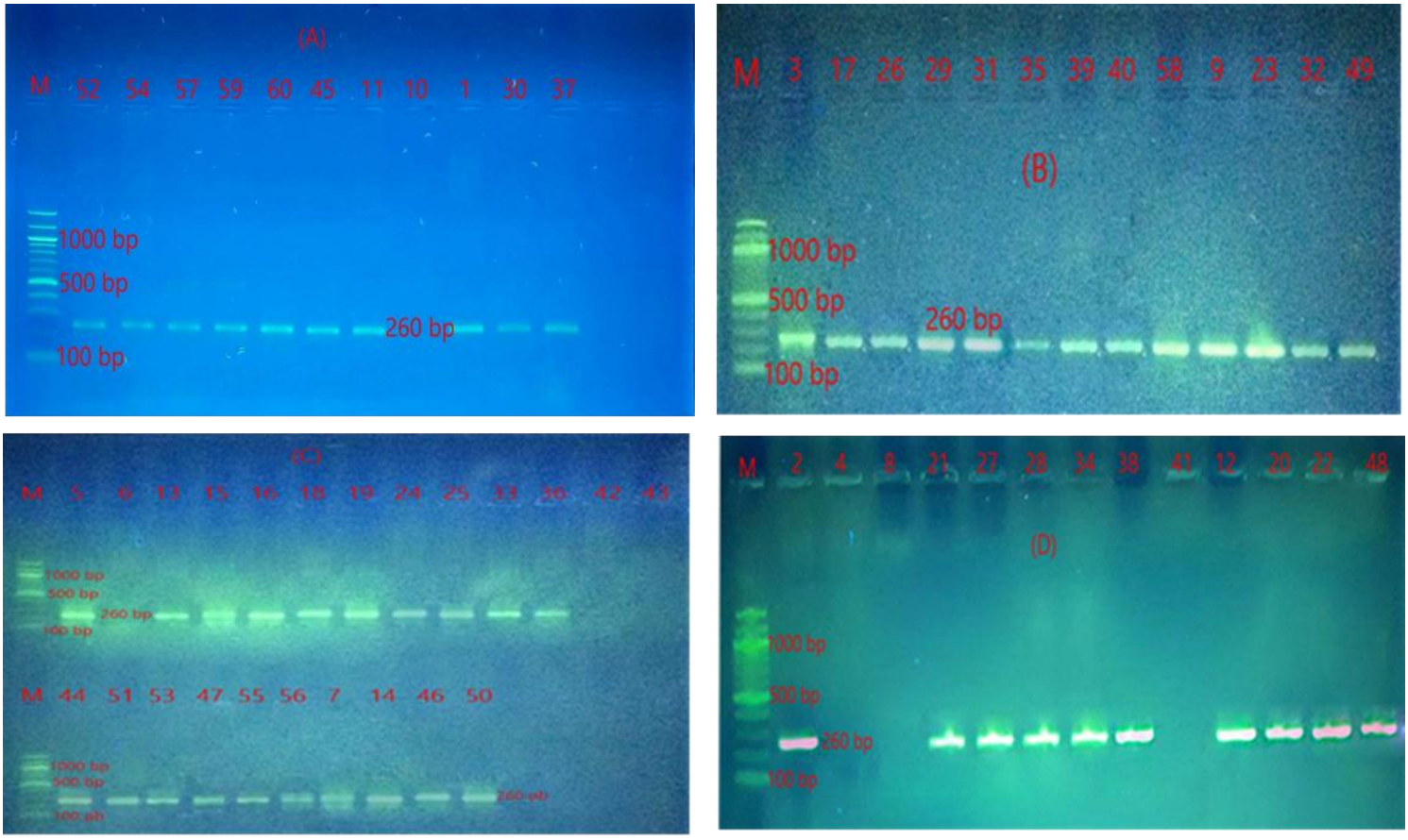


Figure (4.7): Products of polymerase chain reaction performed with *FimH* gene of bacterial isolated. M: 100 bp DNA ladder. (A): *P. mirabillis* (52, 54, 57, 59, 60); *S. aureus* (10, 11, 45); *S. pyogenes* (1, 30, 37). (B): *P. aeruginosa* (3, 17, 26, 29, 31, 35, 39, 40, 58); *K. pneumoniae* (9, 23, 32, 49). (C): *E. coli* (5, 6, 13, 15, 16, 18, 19, 24, 25, 33, 36, 42, 43, 44, 51, 53); *E. faecalis* (47, 55, 56); *S. marcescens* (7, 14, 46, 50). (D): *S. typhi* (2, 4, 8, 21, 27, 28, 34, 38, 41); *Sh. Dysenteriae* (12, 20, 22, 48).

Table (4.1): The prevalence percentage of virulence genes in bacterial isolates

Bacteria	Virulence genes								
	Biofilm			*FimH*			*Iss*		
	Total No.	Positive	%	Total No.	Positive	%	Total No.	Positive	%
E. coli	16	15	93.75	16	14	87.5	16	13	81.25
S. aeruginosa	9	7	77.77	9	9	100	9	9	100
S. typhi	9	9	100	9	5	55.55	9	5	55.55
P. mirabillis	5	5	100	5	5	100	5	5	100
K. pneumoniae	4	4	100	4	4	100	4	4	100
Sh. dysenteriae	4	4	100	4	3	75	4	4	100
S. marcescens	4	4	100	4	4	100	4	4	100
E. faecalis	3	3	100	3	3	100	3	3	100
S. aureus	3	3	100	3	2	66.66	3	2	66.66
S. pyogenes	3	3	100	3	3	100	3	3	100
Total	60	57	95	60	51	85	60	52	86.66

4.1.2.2. Antibiotic resistance genes

The detection of some antibiotics resistance genes (β-lactamase genes) was carried out by using multiplex PCR technique. The results of the present study revealed that bla_{AmpC} and bla_{TEM} were the most prevalence genes among all bacterial isolates, while $bla_{SHV\text{-}5}$ and $bla_{CTX\text{-}M}$ were the less prevalence genes at $P \leq 0.05$ table (4.2). Also, the results showed that the percentages of β-lactamase genes were varied in all bacterial isolates and the highest percentages were observed in bla_{AmpC} and bla_{TEM} in comparison with $bla_{SHV\text{-}5}$ and $bla_{CTX\text{-}M}$ table (4.3).

The positive results of electrophoresis multiplex PCR product of β-lactamase genes were appeared with 670 bp, 531 bp, 410 bp and 247 bp for bla_{AmpC}, bla_{TEM}, $bla_{SHV\text{-}5}$ and $bla_{CTX\text{-}M}$, respectively as shown in the figure (4.8).

Table (4.2): Statistical analysis of the prevalence of β-lactamase genes among bacterial isolates.

Type of gene		Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bla_{AmpC}	*blaTEM*	1.20000	1.01708	.246	-.8627-	3.2627
	blaSHV-5	4.10000*	1.01708	.000	2.0373	6.1627
	blaCTX-M	2.50000*	1.01708	.019	.4373	4.5627
bla_{TEM}	bla_{AmpC}	-1.20000-	1.01708	.246	-3.2627-	.8627
	$bla_{SHV\text{-}5}$	2.90000*	1.01708	.007	.8373	4.9627
	$bla_{CTX\text{-}M}$	1.30000	1.01708	.209	-.7627-	3.3627
$bla_{SHV\text{-}5}$	bla_{AmpC}	-4.10000-*	1.01708	.000	-6.1627-	-2.0373-
	bla_{TEM}	-2.90000-*	1.01708	.007	-4.9627-	-.8373-
	$bla_{CTX\text{-}M}$	-1.60000-	1.01708	.124	-3.6627-	.4627
$bla_{CTX\text{-}M}$	bla_{AmpC}	-2.50000-*	1.01708	.019	-4.5627-	-.4373-
	bla_{TEM}	-1.30000-	1.01708	.209	-3.3627-	.7627
	$bla_{SHV\text{-}5}$	1.60000	1.01708	.124	-.4627-	3.6627

***. The mean difference is significant at the 0.05 level.**

Table (4.3): The prevalence percentage of β-lactamase genes in bacterial isolates

Bacteria	β-lactamase genes											
	bla_{AmpC}			bla_{TEM}			$bla_{SHV\text{-}5}$			$bla_{CTX\text{-}M}$		
	Total No.	positive	%	Total No.	positive	%	Total No.	positive	%	Total No.	positive	%
E. coli	16	15	93.75	16	11	68.75	16	1	6.25	16	4	18.75
S. aeruginosa	9	9	100	9	6	66.66	9	4	44.44	9	6	66.66
S. typhi	9	8	88.88	9	4	44.44	9	1	11.11	9	6	66.66
P. mirabillis	5	5	100	5	5	100	5	2	40	5	3	60
K. pneumoniae	4	3	75	4	3	75	4	1	25	4	0	0
Sh. dysenteriae	4	3	75	4	3	75	4	1	25	4	1	25
S. marcescens	4	4	100	4	4	100	4	0	0	4	2	50
E. faecalis	3	3	100	3	3	100	3	1	33.33	3	2	66.66
S. aureus	3	3	100	3	1	33.33	3	0	0	3	3	100
S. pyogenes	3	3	100	3	3	100	3	1	33.33	3	1	33.33
Total	60	56	93.33	60	43	71.66	60	12	20	60	28	46.66

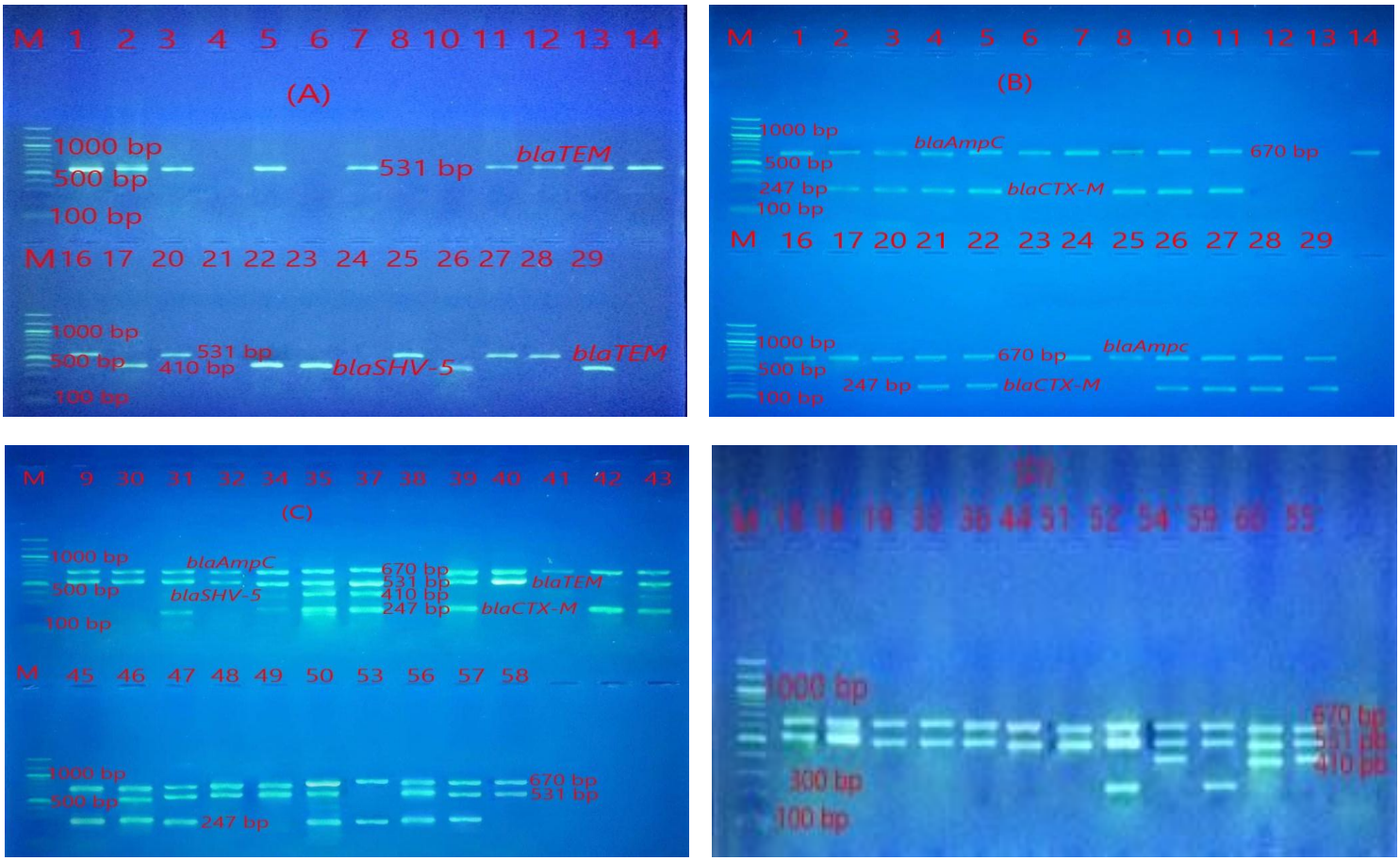


Figure (4.8): Products of polymerase chain reaction performed with β-lactamase genes of bacterial isolated. M: 100 bp DNA ladder. *P. mirabillis* (52, 54, 57, 59, 60); *S. aureus* (10, 11, 45); *S. pyogenes* (1, 30, 37); *P. aeruginosa* (3, 17, 26, 29, 31, 35, 39, 40, 58); *K. pneumoniae* (9, 23, 32, 49); *E. coli* (5, 6, 13, 15, 16, 18, 19, 24, 25, 33, 36, 42, 43, 44, 51, 53); *E. faecalis* (47, 55, 56); *S. marcescens* (7, 14, 46, 50). (D): *S. typhi* (2, 4, 8, 21, 27, 28, 34, 38, 41); *Sh. Dysenteriae* (12, 20, 22, 48).

4.1.3. The correlation between virulence and antibiotics resistance determinants

The results in table (4.4) revealed the profile of virulence factors and antibiotics resistance profile of all bacterial isolates and the statistical analysis of the results showed that there was a strong significance relationship between virulence and antibiotics determinants, where the value of Person correlation was 0.957 at $P \leq 0.01$ as shown in the table (4.5).

Table (4.4): The individual profiles of virulence factors and antibiotic resistance profiles of bacterial isolates.

Isolate No.	Name of isolates	Virulence genes			Antibiotics resistance genes			
		Biofilm	*FimH*	*ISS*	*blaAmpC*	*blaTEM*	*blaSHV-5*	*blaCTX-M*
5	*E. coli*	+		+	+	+		+
6	*E. coli*	+	+		+			
13	*E. coli*	+	+	+		+		
15	*E. coli*	+	+	+	+	+		
16	*E. coli*	+	+	+	+	+		
18	*E. coli*	+	+	+	+	+		
19	*E. coli*	+	+	+	+	+		
24	*E. coli*	+		+	+			
25	*E. coli*	+	+	+	+	+		
33	*E. coli*	+	+	+	+	+		
36	*E. coli*	+	+	+	+			+
42	*E. coli*	+	+		+	+		
43	*E. coli*		+		+		+	+
44	*E. coli*	+	+	+	+	+		
51	*E. coli*	+	+	+	+	+		
53	*E. coli*	+	+	+	+			+
3	*P. aeruginosa*		+	+	+	+		+
17	*P. aeruginosa*		+	+	+		+	
26	*P. aeruginosa*	+	+	+	+		+	+
29	*P. aeruginosa*	+	+	+	+		+	+
31	*P. aeruginosa*	+	+	+	+	+		+
35	*P. aeruginosa*	+	+	+	+	+	+	+
40	*P. aeruginosa*	+	+	+	+	+		
58	*P. aeruginosa*	+	+	+	+	+		
39	*P. aeruginosa*	+	+	+	+	+		+
2	*S. typhi*	+	+		+	+		+
4	*S. typhi*	+			+			+
8	*S. typhi*	+			+			+

21	*S. typhi*	+	+	+	+			+
27	*S. typhi*	+	+	+	+	+		+
28	*S. typhi*	+	+	+	+	+		+
34	*S. typhi*	+		+	+	+	+	+
38	*S. typhi*	+	+	+				
41	*S. typhi*	+	+		+			
52	*P. mirabillis*	+	+	+	+	+		+
54	*P. mirabillis*	+	+	+	+	+	+	
57	*P. mirabillis*	+	+	+	+	+		+
59	*P. mirabillis*	+	+	+	+	+		+
60	*P. mirabillis*	+	+	+	+	+	+	
9	*K. pneumoniae*	+	+	+	+	+		
23	*K. pneumoniae*	+	+	+			+	
32	*K. pneumoniae*	+	+	+	+	+		
49	*K. pneumoniae*	+	+	+	+	+		
12	*Sh. dysenteriae*	+		+		+		
20	*Sh. dysenteriae*	+	+	+	+	+		
22	*Sh. dysenteriae*	+	+	+	+		+	+
48	*Sh. dysenteriae*	+	+	+	+	+		
7	*S. marcescens*	+	+	+	+	+		
14	*S. marcescens*	+	+	+	+	+	+	
46	*S. marcescens*	+	+	+	+	+		+
50	*S. marcescens*	+	+	+	+	+		+
47	*E. faecalis*	+	+	+	+	+		+
55	*E. faecalis*	+	+	+	+	+	+	
56	*E. faecalis*	+	+	+	+	+		+
10	*S. aureus*	+			+			+
11	*S. aureus*	+	+	+	+	+		+
45	*S. aureus*	+	+	+	+			+
1	*S. pyogenes*	+	+	+	+	+		
30	*S. pyogenes*	+	+	+	+	+		
37	*S. pyogenes*	+	+	+	+	+	+	+

Table (4.5): The correlation between virulence and antibiotics determinants of bacterial isolates.

Correlations

		Virulence	Antibiotics
Virulence	Pearson Correlation	1	.957**
	Sig. (2-tailed)		.000
	N	10	10
Antibiotics	Pearson Correlation	.957**	1
	Sig. (2-tailed)	.000	
	N	10	10

****. Correlation is significant at the 0.01 level (2-tailed).**

4.2. Discussion

4.2.1. Phenotype and genotype results

Virulence encompasses not only the ability of bacteria to cause disease in the host (i.e., degree of pathogenicity) but also the ability of bacteria to infiltrate and colonize a host. In the late 1980s and early 1990s, virulence was thought of as a pathogen centered attribute and the concept of pathogenicity islands of genetic virulence factors was introduced in the late 1990s [57]. Genetic virulence factors can regulate physical attributes of the bacteria such as flagella, curli, fimbriae, adhesions, biofilm, [58] or biochemical factors, including host cell surface modifying enzymes, toxins, and antibiotics to provide a competitive advantage.

The current study showed that the biofilm formation was the most virulence factors appeared in all bacterial isolates followed by *Iss* and *FimH* and these results were similar to the results obtained from many authors [59].

A majority of the investigated isolates in the present study were in vitro positive for biofilm production. The prevalence of biofilm production was thus higher than reported in another study: 17% for fecal strains, 43% for strains isolated from patients with cystitis, 40% for pyelonephritis and 42 for bacteremia

E. coli isolates [59]. On the other hand, the same authors reported a high, 63%, prevalence of biofilm formation among strains from patients with prostatitis.

Biofilms are microbial communities of organisms adherent to each other and/or a target surface. Biofilm formation protects bacteria from hydrodynamic flow conditions, for example in the urinary tract, and against phagocytosis and host defense mechanisms, as well as antibiotics [60]. More than 50% of all bacterial infections reported involve biofilm formation [61]. A cascade of several precisely, tightly regulated events are required for proper biofilm formation.

In addition, the latter study showed that haemolysin and type 1 fimbriae expression were significantly associated with biofilm production. Type 1 fimbriae, which promote adhesion to host epithelial cells, have been found to be important in the initial steps of biofilm formation [62] and these results were agreement with the results of the current study.

Hemolysin production is associated with pathogenicity of the organism [63]. In the present study, there were different isolates in different percentages produced hemolysin. Hemolysin production as a virulence factor by urinary isolates has been shown by previous workers [63]. It has been suggested that colonization with hemolytic strains is more likely to develop into urinary tract infections.

The prevalence of *Iss* among bacterial isolates was in the second stage after biofilm and this results showed the importance of *Iss* gene in the pathogenicity of bacterial isolates. Normal serum possesses bactericidal activity against a wide range of gram-negative bacteria [64]. The pathogenicity of these bacteria is partly a function of their ability to evade the bactericidal effect of serum, which is mediated by the complement cascade. Commensal microorganisms are generally vulnerable to the bactericidal effect of serum, while nosocomial bacteria tend to be much more serum resistant [65].

There is a strong correlation between serum resistance and the ability of a variety of gram-negative bacteria to invade and survive in human blood stream. A previous study has shown that serum resistance is important in the pathogenesis of symptomatic UTI, regardless of the severity [63]. The pathogenesis of urinary tract infections depends of the *E. coli* skills to adhere, persist and multiply in the host [66]. The genes involved in bacteria adherence detected in the present study were *pap* and *fimH*.

Also, the results of the current study possessed differences between phenotype and genotype as respect to individual isolates. The detection rates were higher for all tests when genotype assays were used this may be attributed to the DNA amplification by PCR technique is rarely yielded false negative results because of this technique is precise and more accurate than phenotype technique [67].

The results of antibiotics resistance determinants revealed that bla_{AmpC} and bla_{TEM} were the most prevalence genes in all bacterial isolates and these results were similar with many previous studies and also, different with many previous studied [68, 69].

The prevalence and type of *ESBLs* may vary from one geographic region to other. For e.g. in China *TEM*-type were found to be most prevalent *ESBLs* among the *E. coli* (ESBL producing strains) followed by *SHV* and then *CTX*-M-type enzymes [70]. Another report from Canada shows SHV as main group of ESBLs in *E. coli* and only 6% of *ESBL* producers were found to carry blaTEM and *blaCTX*-M [71]. Group 2 *CTX*-M enzymes are the most prevalent *CTX*-M enzymes in parts of South America and of Israel [72] while group 9 enzymes were found to be prevalent in Spain [73].

4.2.2. The correlation between virulence and antibiotics resistance determinants

The present study showed a strong relationship between virulence and antibiotics resistance determinants. This result was similar to many previous studies [74, 75, 76].

There was strong relationship between resistance of antimicrobials and prevalence of some virulence factors in bacteria. Virulence factors such as capsular polysaccharides, outer membrane lipoproteins and biofilms play an important role in protecting bacteria from antimicrobials exposure when compared with those do not have these virulence factors. Bacteria with biofilm forming are generally more resistant to many antimicrobials [77]. Capsular polysaccharides, outer membrane lipoproteins and biofilms act as biodegradable effect on antimicrobials. The β-lactamase enzymes are secreted and maintain their activity inside of biofilm matrix and decompose β-lactam antibiotics before reach to the bacterial cells [77]. Antimicrobials penetration inside of capsular polysaccharides, outer membrane and biofilms could be blocked by other factors, such as the presence of surfaces with negatively-charged, particularly for large polar molecules such as aminoglycosides with positively charged. Also, trace amount of metabolic rates and limited oxygen are may be important factors contributing to increasing resistance to aminoglycosides, β-lactamase, fluoroquinolones and cephalosporins [78].

The resistance–virulence link is complex, considering the diversity of antimicrobial resistance genes, virulence factors, bacterial species and hosts. The correlations between resistance spectrums, virulence factors, and recurrence rates are of great clinical value for clinical diagnosis, treatment, and predictive prognosis of recurrent of different infections including UTI [79].

Conclusions and Recommendations

Conclusions

1) All bacterial isolates showed the ability to produce more than one virulence factor and the prevalence percentages of these virulence factors were different as a respect to the genus of isolates and individuals isolates of the same genus.

2) The biofilm was the most prevalence among bacterial isolates followed by *Iss* and *FimH*.

3) All bacterial isolates possessed antibiotics resistance profile for β-lactamase genes with different percentages.

4) bla_{AmpC} and bla_{TEM} were significantly spread among bacterial isolates compare with $bla_{SHV\text{-}5}$ and $bla_{CTX\text{-}M}$.

5) The current study established a significant correlation between resistance and virulence profile could provide valuable input about the clinical evolution and recurrence rates of different infections.

Recommendations

1) More in depth molecular studies on the genetic support of antimicrobial resistance and virulence determinants are sorely needed to fully understand the interplay of resistance and virulence genes, specially the role of the phylogenetic background of the bacterial isolates in antibiotics resistance profile.

2) Investigate the etiology and local resistance patterns of pathogenic bacteria, which is crucial for determining appropriate empirical antibiotic treatment in patients with different infections.

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