بسم الله الرحمن الرحيم

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Molecular Characterization of Ciprofloxacin Resistance DNA *gyrA* and *gyrB*, *parC* and *parE* genes among *Proteus mirabilis* Isolated from Urine Specimens in Khartoum state- Sudan

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بسم الله الرحمن الرحيم

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صدق الله العظيم

Dedication

This work is dedicated to my

wonderful daughter

The symbol of love and giving

To my Parents

Who gave me Light

To my Teachers

Who taught me wrong from Right

To my brother, sisters,

Who always with me

To Those Who Have,

And Always Will,

Stand Beside me

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ABSTRACT

Background: Urinary tract infections (UTI) are major health problems affecting millions of people each year. As an opportunistic pathogen *Proteus mirabilis* causes urinary tract infections, ciprofloxacin is a recommended drug for the treatment of UTIs, but a progressive increase in fluoroquinolone resistance has been seen in clinical isolates. The study was a qualitative study, aimed to highlight the importance of using conventional and molecular techniques in the detection of different mutations within the genome of *Proteus mirabilis* isolates.

Methods: In this study a total of (3895) specimens were collected from patients with symptoms of UTIs attending different hospitals in Khartoum State during the period from June 2016 to May 2017. Midstream urine samples were collected and cultured for UTI diagnosis and ciprofloxacin susceptibility. Then polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing was performed for detection of *GyrA*, *GyrB*, *ParC* and *ParE* genes mutations of isolated *P. mirabilis*.

Results: The findings of this study suggested high prevalence of UTIs among females (53.5%) compared to males (46.5%). *P. mirabilis* was isolated from (3.1%) patients, with different ratio (females, 55:males, 45), occurred highest in the age group (11 – 49 years).

UTI is commonly caused by a range of pathogens, but most commonly by *Escherichia coli* (56.1%) followed by *Klebsiella pneumoniae* (18.0%), *Enterococcus faecalis* (9.9%), *Pseudomonas aeruginosa* (8.9%), *Proteus mirabilis* (3.1%), *Proteus vulgaris* (2.2%), *Citrobacter* spp. (0.8%), *Staphylococcus epidermidis* (0.6%) and *Staphylococcus aureus* (0.4%). The target

urinary isolates reflected high resistance to ciprofloxacin (71.7%), whilst *P. mirabilis* shown (30%) resistant to ciprofloxacin.

Mutations associated with ciprofloxacin resistance *P. mirabilis* in *GyrA* (Ser 83 to Ile) and *ParC* (Ser 81 to Ile). Also it revealed silent mutations at following codons of *GyrB* (474 leucine, 585 valine, 612 histidine and 639 asparagine) and *ParE* (469 isoleucine, 531 aspartic and 533 glycine).

Conclusion: Only one or two mutations in both *GyrA* and *ParC* genes of *Proteus mirabilis* are necessary to obtain resistance to ciprofloxacin, *ParC* is important as *GyrA* to cause resistance susceptibility to ciprofloxacin in *Proteus mirabilis* and In *Proteus mirabilis* the silent mutation in QRDR regions is not enough for ciprofloxacin resistance.

ملخص الدراسة

خلفية الدراسة : يعتبر التهاب المسالك البولية من اكبر المشاكل الصحية التي تصيب أكثر من مليون شخص سنويا. تسبب المتقلبه الرائعه التهاب المسالك البولية كنوع من الاصابة الانتهازية، يعتبر السيبروفلوكساسين احد الادوية الموصى بها لعلاج التهاب المسالك البولية، ولكن لوحظ زيادة مطردة في مقاومة الفلوروكينولون في البكتريات المعزوله سريريا. كانت الدراسة عبارة عن دراسة نوعية ، تهدف إلى تسليط الضوء على أهمية استخدام التقنيات التقليدية والجزيئية في الكشف عن الطفرات المختلفة داخل الجينوم المتقلبة الرائعة.

طرق الدراسة: في هذه الدراسة تم جمع (٣٨٩٥) عينه من مرضي تظهرعليهم أعراض التهاب المسالك البولية من مستشفيات مختلفة داخل ولاية الخرطوم في الفتره من يوليو ٢٠١٦ الي مايو ٢٠١٧. تم جمع العينات من منتصف البول وزرعت لتشخيص اللتهاب المسالك البولية و اختبار حساسية السيبروفلوكساين. ثم تم اجراء تفاعل البلمرة المتسلسل PCR و RFLP و التسلسل الجيني للكشف عن الطفرات الجينية GyrA، GIRB، ParC و ParE من المتقلبة الرائعه معزولة.

النتائج: تشير النتائج إلي ارتفاع معدل انتشارعدوى المسالك البولية بين الإناث مقارنة مع الذكور بنسب (٥٣.٥% : ٤٦.٥%) كما تم عزل المتقلبة الرائعة من (٣.١%) مريض، بنسبة مختلفة (٥٥ إناث :٤٥ ذكور)، اعلي معدلات للإصابة في مجموع الأعمار من (١١-٤٩ سنه).

تحدث عدوى المسالك البولية عادة عن طريق مجموعة من مسببات الأمراض، ولكن أكدت النتائج وجود الاشرشيا القولونية بين المرضى السودانيين بنسبة عالية (٥٦.١%) تليها الكليبسيلا الرئوية (١٨.٠ %) ، المكورة المعوية البرازية (٩.٩%)، الزائفة الزنجارية (٨.٩%)، المتقلبة الرائعة (٣.١%)، المتقلبة الشائعة (٢.٢%)، أنواع الليمونية (٠.٨%)، ،الكرويّة العنقودية الجلديّة (٠.٦%) وأقل نسبة للبكتيريا المعزولة كانت للمكورة العنقودية الذهبية (٠.٤%)، البكتريا المعزولة المستهدفه في دراستنا عالية المقاومة للسيبروفلوكساسين (٧١.٧%)، في حين ان(٣٠%) من المتقلبة الرائعة مقاومة للسيبروفلوكساسين.

تم تحديد الطفرات المرتبطة بمقاومة السيبروفلوكساسين في المتقلبة الرائعة في*GyrA* (تغير السيرين في الكودون ٨٣ إلي ايزوليوسين) و(السيرين في الكودون ٨١ إلي ايزوليوسين) من *ParC*. وكشف عن طفرات صامتة في الكودونات(٤٧٤ليوسين، ٥٨٥ فالين ، ٦١٢ هيستدين

و٦٣٩الأسباراجين) من*GyrB* و (٤٦٩ ايزوليوسين، ٥٣١ اسبارتيك و ٥٣٣ جلايسين) من*ParE*.

الخلاصة: وجود طفره جينيه واحده او اثنين في *GyrA* او *ParC* من المتقلبه الرائعة مهم لايجاد مقاومه لليبروفلوكساسين، يعتبر *ParC* مهم مثل*GyrA*حيث يقلل حساسية البكتريا للسيبروفلوكساسين وكما ان الطفرة الصامتة في مناطق QRDR لا تكفي لمقاومة السيبروفلوكساسين في المتقلبة الرائعة.

PREFACE

Part of the project's findings was published in peer-reviewed journals:

1. Randa H Abdelkreem, Leila M Abdelgadeir and Mogahid M Elhassan (2018). Ciprofloxacin Susceptibility of Proteus Mirabilis Isolated from Sudanese Patients with Urinary Tract Infections. IOSR Journal of Dental and Medical Sciences. 17 (4): 85-87.

2. Randa H abdelkreem, Leila M Abdelgadeir, Miskelyemen A. Elmekki, Husham N. Altayb and Mogahid M Elhassan (2018). Characterization of Ciprofloxacin Resistant Proteus mirabilis and Evaluation the Effects of gyrA and gyrB, parC and parE Mutations in Quinolones Resistant Urinary Isolates (under processing).

Also the following sequences have been submitted to MBL/GENEBANK

Sample	Accession numbers	Target gene	Amino acid change	
			Amino acid	Nucleotide
1A	MH310924	*GyrA*	-	-
3A	MH310925	*GyrA*	Ser 83 Ile	AGT-ATT
8A	MH310926	*GyrA*	-	-
1B	MH310921	*GyrB*	Lus 474 Lus	TTA -TTG
			Val 585 Val	GTT -GTC
3B	MH310922	*GyrB*	Lus 474 Lus	TTA -TTG
			Val 585 Val	GTT -GTC
8B	MH310923	*GyrB*	Lus 474 Lus	TTA -TTG
			His 612 His	CAC-CAT
			Asn 639 Asn	AAT AAC
1C	MH310927	*ParC*	Ser 84 Ile	AGT-ATT
3C	MH310928	*ParC*	Ser 84 Ile	AGT-ATT
			Pro 116 Pro	CCA CCT
8C	MH310929	*ParC*	His 81 His	CAC CAT
1E	MH310930	*ParE*	-	-
3E	MH310931	*ParE*	Ile 469 Ile	ATC -ATT
			Asp 531 Asp	GAC-GAT
8E	MH310932	*ParE*	Ile 469 Ile	ATC -ATT
			Asp 531 Asp	GAC-GAT
			Glu 533 Glu	GGT-GGA

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LIST OF ABBREVIATIONS

A. baumannii	*Acinetobacter baumannii*
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATF	Ambient-temperature fimbriae
ATP	Adenosine triphosphate
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CAP	Catabolite-activator-protein
CLED	Cystine Lactose Electrolyte Deficient
CTD	C-terminal domain
DNA	Dioxyrbonuclic acid
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. tarda	*Edwardsiella tarda*
Gly	Glycine
GyrA	Subunit A of DNA gyrase
GyrB	Subunit B of DNA gyrase
His	Histidine
HpmA	Hemolysin P. *mirabilis* A
hrs	Hours
HTH	Helix Turn Helix
Ile	Isoleucine
K. pneumoniae	*Klebsiella pneumoniae*
kDa	Kilo Dalton
KIA	Kligler Iron Agar
leu	Leucine
LPS	Lipopolysaccharide
M. bovis	*Mycoplasma bovis*
MR/P	Mannose-resistant/Proteus-like
N. gonorrhoeae	*Neisseria gonorrhoeae*
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nano gram
NTD	N-terminal domain
OMPs	Outer Membrane Proteins
P. mirabilis	*Proteus mirabilis*
P. vulgaris	*Proteus vulgaris*
ParC	subunits A of topoisomerase IV
ParE	subunits B of topoisomerase IV

PCR	Polymerase Chain Reaction
PMF	*P. mirabilis* fimbriae
PMP	*Proteus mirabilis* P-like pili
Pro	Proline
Ps. aeruginosa	*Pseudomonas aeruginosa*
Pta	Proteus toxic agglutinin
QRDRs	Quinolone resistance-determining regions
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
S. saprophyticus	*Staphylococcus saprophyticus*
S. aureus	*Staphylococcus aureus*
Ser	Serine
TBE	Tris base Boric acid EDTA
TBE buffer	Tris base, Boric acid and EDTA buffer
TCA	Tri carboxylic acid
TNF	Tumor necrosis factor
Tyr	Tyrosine
UCA	Uroepithelial cell adhesion
UTIs	Urinary tract infections
UV	Ultra Violet
Val	Valine
WFI	Water for injection
µL	Micro liter

CHAPTER ONE
INTRODUCTION AND OBJECTIVES

1.1 Introduction

Urinary tract infections (UTIs) are serious health problems affecting millions of people each year. They are considered as the second most common type of infection in the body (Murtada *et al*., 2014). *Proteus mirabilis* is one causes of urinary tract infections (UTIs) among members of the *Enterobacteriaceae* family (Rozalski *et al.,* 1997), As an opportunistic pathogen, *P. mirabilis* causes urinary tract infections, wounds, burns, respiratory tract, and other sites infections (Weigel *et al*., 2002). Which are often persistent and difficult to treat, and is also an important cause of nosocomial infections (Rozalski *et al.,* 1997).

The treatment of UTIs differs according to the age of the patient, sex, underlying disease, infectious agent and whether there is lower or upper urinary tract involvement. Ciprofloxacin is a recommended drug for the treatment of UTIs (Endimiani *et al.,* 2005). Though wild-type strains of *P. mirabilis* are usually susceptible to fluoroquinolones (Endimiani *et al.,* 2005; Hernandez *et al*., 2000), but a progressive increase in fluoroquinolone resistance has been seen in clinical isolates (Hernandez *et al*., 2000; Kim *et al*., 2004).

Fluoroquinolones and earlier quinolones are novel among antimicrobial agents in clinical use because they directly inhibit deoxyribonucleic acid (DNA) synthesis. Inhibition appears to occur by interaction of the drug with complexes composed of DNA and either of the two target enzymes, DNA gyrase and topoisomerase IV. These enzymes are structurally related to each other, both being tetrameric with pairs of two different subunits. The *GyrA* and *GyrB* subunits of DNA gyrase are

respectively homologous with the *ParC* and *ParE* subunits of topoisomerase IV. Both enzymes are type 2 topoisomerases, which act by breaking both strands of a segment of DNA, passing another segment through the break, and then resealing the break. For DNA gyrase, this topoisomerization reaction results in removal of DNA supercoils, thus affecting the negative supercoiling of DNA necessary to initiate DNA replication and removes positive supercoils that accumulate before an advancing replication fork. For topoisomerase IV, the topoisomerization reaction results in separation of the interlocking of daughter DNA strands that develop during replication, this facilitates the segregation of daughter DNA molecules into daughter cells. In both cases, fluoroquinolones appear to trap the enzyme on DNA during the topoisomerization reaction, forming a physical barrier to the movement of the replication fork (Hiasa *et al*., 1996), Ribonucleic acid (RNA) polymerase, and DNA helicase. The collision of the replication fork with these trapped complexes triggers other poorly defined events within the cell that ultimately result in cell death (Willmott *et al.,* 1994; Shea and Hiasa*,* 1999).

The mechanisms of fluoroquinolone resistance include one or two of the three main mechanistic categories, alterations in the drug target, and alterations in the permeation of the drug to reach its target (Wetzstein *et al.,* 1997). The primary mechanisms of resistance to fluoroquinolones are mutations that result in alteration of the target proteins, DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*), and decreased intracellular drug accumulation due to drug efflux or changes in outer membrane proteins. In several species of *Enterobacteriaceae*, decreased susceptibility or resistance to fluoroquinolones is associated with specific point mutations in gyrA. Additional mutations in the gyrase or topoisomerase IV genes contribute to higher levels of resistance (Weigel *et al*., 2002).

1.2 Hypothesis of the Study

It has been postulated that the active-site region of DNA gyrase, and by homology of topoisomerase IV, may constitute the region of quinolone binding, since *GyrA* mutations result in decreased drug binding. No structural data on topoisomerase-DNA-quinolone complexes have, however, been reported. Therefore, the exact mode of binding of quinolones to the enzyme-DNA complex remains unknown.

1.3 Rational

UTIs are serious health problems affecting millions of people each year. They are considered as the second most common type of infection in the body. Misuse of antibiotics in developing countries such as Sudan, encourages the phenomenon of drug resistance. Whilst ciprofloxacin is a recommended drug for the treatment of UTIs in Sudan. A progressive increase in resistance to ciprofloxacin has been seen in clinical isolates of *Proteus mirabilis*.

1.4 Research Questions

- What is a real situation of antimicrobial susceptibility pattern of *Proteus mirabilis* among Sudanese patients?
- Which one of the DNA *GyrA* and *GyrB*, *ParC* and *ParE* genes is commonly responsible for the ciprofloxacin resistance in Sudan?
- Is there any detectable mutation (s) in any of the study genes?

1.5 Objectives

1.5.1 General objective

Molecular characterization of ciprofloxacin resistance genes among *Proteus mirabilis* isolated from Sudanese patents.

1.5.2 Specific objective

- To isolate and identify different pathogenic bacteria that caused UTIs among Sudanese patients.
- To estimate the frequency of *Proteus mirabilis* among UTI isolates against empirical antibiotics by using conventional methods.
- Identify and type *GyrA*, *GyrB*, *ParC* and *ParE* in *Proteus mirabilis* isolates by using PCR technique.
- To determine different mutations (if present) among *GyrA* and *GyrB*, *ParC* and *ParE* by DNA sequencing.
- To compare the results of sensitivity pattern obtained by PCR with Modified Kirby Bauer method.

CHAPTER TWO
Literature Review

2.1 The Genus *Proteus*

2.1.1 Definition

Proteus mirabilis is a small gram negative bacillus and a facultative anaerobe, it ferments maltose, and inability to ferment lactose. *P.mirabilis* has the ability to elongate itself and secrete a polysaccharide when in contact with solid surfaces, making it extremely motile on items such as medical equipment. *P. mirabilis* has ability to swarm over the surface of media, this characteristic makes them readily recognizable in the laboratory. It differs from other *Enterobacteriaceae* in the production of a very strong urease, which aids their rapid identification. It also leads to production of urinary stones and produces alkalinity and an ammonia odor to the urine (Ohara *et al*.,2000).

P. mirabilis is part of the *Enterobacteriaceae* family, it is part of the normal flora of the human gastrointestinal tract of mammals. It can also be found free living in water and soil. When this organism, however, enters the urinary tract, wounds, or the lungs it can become opportunistic pathogenic. *P. mirabilis* commonly causes UTIs and the formation of stones. (Jacobsen and Shirtliff, 2011).

2.1.2 Historical Background

P. mirabilis was first discovered by a German pathologist named Gustav Hauser in 1885. He named this genus *Proteus*, after the character in Homer's the Odyssey that was good at changing shape and evading being questioned. The genus originally had two species: *P. mirabilis* and *P. vulgaris*, both first described by Hauser in 1885. He noted the swarming nature of the organisms and divided the strains into the two

species based on the speed of their ability to liquefy gelatin: *P. vulgaris* liquefies gelatin "rapidly," and *P. mirabilis* does so "more slowly". Hauser also described "*Proteus zenkeri*," which neither swarmed nor liquefied gelatin, but he rescinded this particular finding (7 years) later (Williams and Schwarzhoff, 1978).

2.1.3 Classification

The genus *Proteus* is classified in the phylum proteobacteriaas part of the *Enterobacteriaceae* family. In this family it is placed in the tribe *Proteeae,* together with the genera *Morganella* and *Providencia*. *Proteus* rods are distinguishable from most other genera by their ability to swarm across agar surfaces of solid media. The genus *Proteus* currently consists of five species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, as well as three unnamed *Proteus* genome species (O'hara *et al.*2000; Janda and Abbot 2006). The differentiation of *Proteus mirabilis* rods according to the results of biochemical tests (Do not ferment lactose, rapidly hydrolyze urea (within 4 hours), This is an important early screening test in differentiating enteric pathogens, e.g. *Salmonellae* and *shigellae* from *Proteus*, *P. mirabilis* is a facultative anaerobe. It is able to produce urease enzyme, which then hydrolyzes urea to ammonia and carbon dioxide, the bacterium also produces hydrogen sulfide(Coker *et al*., 2000). The *P. mirabilis* ferment sucrose and ferment glucose with production of gas, *P. mirabilis* produce the enzyme phenylalanine deaminase, which is needed for it to use the amino acid phenylalanine as a carbon and energy source for growth, don't produce indole from the amino acid tryptophan (*P. vulgaris* produce indole from the amino acid tryptophan). Citrate test different strains give different results (Cheesbrough, 2007).

2.1.4 General Properties

P. mirabilis isolated from the intestinal tract of mammals, birds and reptiles. They also are distributed widely in the environment, with reservoirs in soil, polluted water and sewage, where they play an important role in decomposing organic matter of animal origin. Besides the saprophytic mode of life in the natural environment and in the intestines of humans and domestic animals, optimal environmental conditions for the bacterium are a highly alkaline environment. As a facultative anaerobe, *P. mirabilis* does not require oxygen for reproduction and survival (Miguel*et al*., 2010).

P. mirabilis is a facultative anaerobe. It is able to produce urease, which then hydrolyzes urea to ammonia and carbon dioxide, the bacterium also produces hydrogen sulfide (Coker *et al*., 2000).

2.1.5 Toxins and Enzymes

Two toxins encoded by *P. mirabilis* hemolysin HpmA and Proteus toxic agglutinin (Pta). In addition to HpmA and Pta, activity of the enzyme urease also contributes to tissue damage (Greta, 2010).

Hemolysin is a toxin that inserts into target eukaryotic cell membranes forming a pore, causing the efflux of ions and subsequent cell damage (Braun and Focareta,1991). Hemolysin facilitates bacterial spread within the kidney and development of pyelonephritis during ascending UTIs (Lukomski *et al*., 1991).

Pta protein contains active α-domain capable of lysing kidney and bladder cells. *P. mirabilis* negative Pta gene had reduced pathology as well as, a significant colonization defect in the bladder, kidneys and spleen (Alamuri and Mobley 2008; Alamuri *et al*.,2009).

P. mirabilis produces urease, an enzyme that converts urea into ammonia by the following process: $\{(NH_2)_2CO \rightarrow 2NH_3 + CO_2\}$. Infection by

P. mirabilis can therefore be detected by an alkaline urine sample (pH 8 and up) with large amounts of ammonia (Gonzales, 2006).

2. 2 Pathogenicity of *Proteus mirabilis*

2.2.1 Virulence Factors

Like many other pathogens *P. mirabilis* has evolved numerous virulence factors that are important for causing UTI and several of these factors appear to be more important for establishing infection in different areas of the urinary tract. These factors include adherence to host mucosal surfaces, damage and invasion of host tissues, evasion of host immune systems, and iron acquisition. There are, as well, virulence factors that are crucial for successful colonization of the urinary tract (e.g., urease for hydrolyzing urea and flagella for ascending to kidneys through ureters). Expression of these virulence factors is most likely spatially and temporally regulated. The bacterium must gain entry to the urinary tract via the urethra (or indwelling catheter), travel to and colonize the bladder, ascend the ureters to the kidneys, colonize the kidneys and maintain infection. In some cases, the organism may gain access to capillaries and establish bacteremia. These events require the organism to express different adhesions and flagella concordant with morphological changes from vegetative swimmer cell to hyperflagellated swarmer cell, express toxins, and avoid the host immune response (Coker *et al*., 2000).

P. mirabilis is an opportunistic pathogen of the urinary tract in older individuals. It is not a common cause of urinary tract infections in normal hosts, more than (90%) of which are due to *E. coli* infections. However, in patients with structural or functional abnormalities in their urinary tracts or patients with long-term catheterization, up to (44%) of the urinary tract infections are caused by *P. mirabilis*. Due to the production of urease by this organism, infection with *P. mirabilis* not only develops into cystitis and acute pyelonephritis but also causes stone formation in

the bladder and kidneys. This urolithiasis is a hallmark of infection with this organism (Coker *et al*., 2000).

2.2.1.1Flagella

The most distinctive characteristic of *P. mirabilis* and other *Proteus* species is swarming motility. Swarming motility, simply defined, is "the movement of highly elongated and flagellated swarm cells across the surface of a solid medium in periodic cycles of movement and consolidation" Considering the fact that this specialized form of motility was first reported by Hauser in 1885 (Williams and Schwarzhoff, 1978).

P. mirabilis can display two different morphological and physiological forms; one is known as the swimmer cells and the other as swarmer cells. When cultured in broth (referred to as vegetative swimmer cells) are typically (1-2 μm) long; after inoculation onto solid agar medium, the cells undergo a drastic morphological change and they increase to (20-80 μm) in length. As the shorter cells elongate, there is a corresponding increase in the number of flagella per cell; short cells typically have (1-10) flagella, but the number of flagella present on the elongated cells has been estimated to be between (500 - 5,000). In addition, the rate of DNA replication remains constant even though septation is inhibited (Nielubowicz, 2010); thus *P.mirabilis* swarming also requires differentiation into distinct swarm cell morphology. Regulation of the swarm cell differentiation process is not fully understood, but many components have been investigated and recently reviewed. For instance, surface contact and the resulting inhibition of flagellar rotation are critical for swarm cell differentiation in most strains, and a combination of surface contacts and changes in cell wall and lipopolysaccharide composition ultimately promotes activity of the flagellar master regulator and expression of the flagellar genes. Factors that impact temporal regulation of swarming, swarm speed, or overall swarm pattern have also

been identified, such as putrescine and certain fatty acids. There is also an intimate connection between swarming and energy metabolism, as normal swarming requires pathways that generate pyruvate and a complete oxidative tricarboxylic acid (TCA) cycle, even though *P. mirabilis* appears to use anaerobic respiration during swarming. *P. mirabilis* swarming is also influenced by aeration, growth rate, cell density, and the concentration of Sodium chloride (NaCl) or other electrolytes (Chelsie*et al*., 2013). Many ways of inhibiting swarming in cultural plates have been described: these include the physical restriction of movement of *Proteus* cells by increasing the agar concentration to (3-4 %), the prevention of the formation of flagella through the incorporation into the media of ethanol (5.5%), detergents, bile salts or other surface-active agents and the retardation of the cell growth rate by incorporating into the media growth inhibitors. Swarming is inhibited in MacConkey agar by the bile salts, so on this kind of medium *Proteus* strains form discrete colonies (Cheesbrough, 2006).

2.2.1.2Fimbriae and Adherence Ability

Bacterial adhesion to the uroepithelium is an essential step for colonization and infection, particularly in a system of continuous urinary flow. Analysis of the genome sequence of *P. mirabilis* produces several fimbriae and hemagglutinins, including mannose-resistant/Proteus-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), uroepithelial cell adhesion (UCA), ambient-temperature fimbriae (ATF), and *P. mirabilis* P-like pili (PMP) (Pearson*et al*., 2008; Rocha *et al*., 2007).

The MR/P fimbriae (mannose-resistant/Proteus-like) are the best understood and most important fimbriae of *P. mirabilis*. The MR/P gene cluster is constituted by two transcripts mrpABCDEFGHJ (mrp operon) and mrp(Bahrani and Mobley, 1994), Several studies have been demonstrated association of MR/P fimbriae and uropathogenesis in

murine model. Other studies have been demonstrated that this fimbria is immunogenic and can be used as vaccination target (Rocha *et al*., 2007).

The uroepithelial cell adhesion (UCA) is organized as long and flexible rods. The role of these fimbriae in the virulence of *P. mirabilis*, the UCA plays an important role in the colonization of the urinary tract using a UCA mutant and wild-type strains in murine model of infection. The UCA operon contains five genes denominated PMI0532-PMI0536 of the genome sequence of HI4320 (Pearson*et al*., 2008).

The ambient-temperature fimbriae (ATF) are important in ambient *P. mirabilis* life style. This fimbria optimal expression is only in (23°C), (Zunino *et al.,*2000) there is significant difference of ATF mutant strain and wild-type in a murine model of urinary infection. Taken together these fimbriae are not important in *P. mirabilis* host colonization (Massad *et al*.,1994).

The *P. mirabilis* fimbriae (PMF) were isolated and their operon nucleotide sequence was determined by (Massad and Mobley, 1994). The genetic organization of the PMF fimbriae operon revealed five functional genes pmfACDEF (Massad and Mobley., 1994; Massad *et al*.,1994). The important role of this fimbria in *P. mirabilis* colonization of bladder and kidneys (Zunino *et al*., 2003).

The *P. mirabilis* P-like pili (PMP) were characterized from *P. mirabilis* strain isolated from a dog with urinary infection (Gaastra *et al*.,1996). PMP are present in the genome of human *P. mirabilis* uropathogenic strain HI4320. The genetic operon organization contains (9 genes PMI2216-PMI2224) (Pearson*et al*., 2008).

2.2.1.3 Outer Membrane Proteins

P. mirabilis outer membrane proteins (OMPs) play a critical role in the host–pathogen interaction, both by protecting bacteria from a hostile environment and by promoting their virulence (Finlay and Falkow, 1997).

The OMPs are key players in bacterial adaptation to different environment conditions as they control the nutrient passage across the membrane and excretion of toxic compounds. The OMPs expression is regulated by environmental signals, as for example, the abundance or lack of specific nutrients, which makes them essential for the adaptation to host niches (Lin *et al.,* 2002).

Outer membrane proteins (OMP) possess immunogenic properties and mitogenic activity for B cells, OM lipoproteins and their synthetic analogs function as adjuvants and can also activate macrophages to produce tumor necrosis factor (TNF). The OM of *P. mirabilis* contains three major proteins of (39.0, 36.0, and 17.0 kDa). The (39-kDa) protein was identified as the OmpA protein, and the (36-kDa) protein appeared to be a peptidoglycan-associated matrix protein (Miguel *et al*., 2010).

2.2.1.4 Lipopolysaccharide (O-antigen, Endotoxin)

Lipopolysaccharide (LPS) are endotoxins, well-known of gram-negative bacteria, which cause a broad spectrum of pathophysiological effects such as fever, hypotension, disseminated intravascular coagulation, and lethal shock. Endotoxin can be released from cell surfaces of bacteria during their multiplication, lysis and death (Antoni *et al*., 1997).

Proteus is anantigenically heterogeneous genus, principally because of structural differences of its O-specific polysaccharide chain of LPS (O antigen), as well as its H antigen. The chemical classification of *P. mirabilis* and *P. vulgaris* LPS in to (16) chemotypes (Sidorczyk *et al*., 1975).

Lipopolysaccharide bacterial surface antigen is recognized by specific antibodies produced by the host defense system. LPS from the S form of pathogenic bacteria contributes to their resistance against bactericidal action of serum and intracellular killing by phagocytes (Antoni *et al*., 1997).

2.2.1.5 Capsule Antigens

The capsule structure termed as slime material or glycocalyx (highly hydrated polymers present on the surface of bacteria), was demonstrated to be a potential pathogenic factor of *Proteus* strains because of its positive effect on struvite crystal growth and stone formation. *P. mirabilis* (O6 and O57) and *P. vulgaris* (O19) could synthesize a capsule antigen structure identical to the O-specific chain of their LPS. The acidic character of *Proteus* due to the presence of uronic acids, pyruvic acid, or phosphate groups, enabled them to bind metal cations (e.g., Mg21) via electrostatic interactions (Antoni*et al*., 1997; Dumanski *et al*., 1994).

2.2.1.6 Hemolysin

Hemolysin is a toxin that inserts into target eukaryotic cell membranes forming a pore, causing the efflux of ions and subsequent cell damage (Braun and Focareta,1991). Hemolysin facilitates bacterial spread within the kidney and development of pyelonephritis during ascending UTIs. The hemolysin genes of *P. mirabilis* are two-partner secretion system (hpmA and hpmB). HpmB transports and activates HpmA. HpmA is found in the periplasm, while HpmB is probably found in the outer membrane to be located, participating in the secretion process of HpmA(Lukomski *et al*., 1991).

2.2.1.7 Proteus Toxic Agglutinin (Pta)

P. mirabilis Pta protein is outer-membrane autotransporter that mediates cell–cell aggregation and also contains a catalytically active α-domain capable of lysing kidney and bladder cells. *P. mirabilis* negative Pta gene had reduced pathology as well as, a significant colonization defect in the bladder, kidneys and spleen(Alamuri and Mobley 2008; Alamuri *et al*.,2009).

2.2.1.8 Urease Enzyme

Urease is very important in *P. mirabilis* pathogenesis. This enzyme catalyzes the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary (Coker *et al*., 2000). The urea-inducible urease gene cluster (urea RDABCEFG) encodes a multimeric nickel metalloenzyme that hydrolyzing urea to ammonia and carbon dioxide, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine (stone formation). This pH alteration is important during *P. mirabilis* catheter colonization, facilitating the bacterial adherence and formation of biofilm incrustation(Coker *et al*., 2000; Cristiani and Sergio, 2014). Stone formation is a hallmark of *P. mirabilis* infection, supplying a number of advantage including, the host immune system protection, blockage of the ureters, ammonia toxicity to host cells, and direct tissue damage. These facts lead to a protective and nutrient-rich environmental niche for the microorganism (Cristiani and Sergio, 2014).

2.2.1.9Metalloproteinase (ZapA)

The bacteria persistence in the host must evasion of innate and adaptive immune responses. *P. mirabilis* presents several evasion mechanisms. *P. mirabilis* encodes a metalloproteinase (ZapA), that cleaves serum and secretory immuno-globulin (A1 IgA1, IgA2 and IgG) (Walker *et al*.,1999). Demonstrated that ZapA mutant ion results in a dramatic decrease in the recovery of bacteria from the urine, bladder and kidneys. This microorganism has ability to vary expression of MR/P fimbriae and flagella, thus tricking the immune system (Bahrani and Mobley.,1994). As already mentioned, the stone formation is characteristic of uropathogenesis of *P. mirabilis*. This event contributes to persistence by causing retention of urine, generating a reservoir of bacteria, preventing wash-out and evasion of immune system (Sabbuba*et al*., 2003)**.**

2.2.2 Transmission

P. mirabilis can be found as a free-living microbe in soil and water. The organism is also normally found in the gastrointestinal tract of humans. *P. mirabilis* has access to the bladder by infecting the periurethral area and causes UTIs primarily through indwelling catheters. Usually the urinary tract can wash out the microbe before it accumulates, but the catheter prevents this from happening. *P. mirabilis* can then adhere to the insides and outsides of the catheter, forming biofilm communities. Once established, these microbes pass through the urethra via swarming motility to the bladder. *P. mirabilis* binds to bladder epithelial cells where it eventually colonizes (Coker *et al*., 2000). *P. mirabilis* infection can also lead to the production of kidney and bladder stones. The bacteria colonize the stones as they form, making them less accessible to antibiotic attack (Pearson *et al*., 2008).

2.2.3 Pathogenesis

Pathogenicity of *P. mirabilis* is accomplished in the following two steps. First the microorganism needs to colonize the urinary tract and second, the microorganism needs to successfully evade host defenses. Colonization of the urinary tract is done by using two of the four types of fimbriae called Mannose-resistant fimbriae (MRF) and *P. mirabilis* fimbriae (PMF). The importance of the MRF was determined in a study done at the Mobley lab at the University of Michigan Medical School. In their study they were able to successfully produce a nasal vaccine against MRF that worked in mice (Janson, 2003).

There are four possible mechanisms by which *P. mirabilis* can use to evade the host defenses. The first is production of an IgA-degrading protease which functions to cleave the secretory IgA. IgA is released by the host in an initial response to infection (Janson, 2003). The second immune system evasion mechanism is through three unique flagellin

genes, which have been shown to recombine and form novel flagella capable of tricking the host's defenses (Belas *et al.,*2003). The third is through expression of the MR/P fimbriae is immunogenic and can be used as vaccination target. They go through a process called phase variation by which the expression of flagella is found in some cells but not in others of the same population. The fourth mechanism is the urease-mediated stone formation. Production of ammonia by the action of urease results in stone formation, and these stones in turn, help protect the bacteria (Janson, 2003).

Urease and hemolysin are known to cause damage to host epithelial cells. As mentioned above, urease can damage host epithelial cells through the formation of stones. Hemolysin damages cells because of its property as a potent cytotoxin (Janson, 2003).

2.2.4 Clinical Feature

Patients may present with urethritis, cystitis, prostatitis, or pyelonephritis. Chronic, recurring stones may be an indication of chronic infection. Urethritis are usually mild and may be dismissed by the patient. Women present with dysuria, pyuria, and increased frequency of urination. Presenting symptoms in males are usually mild and may include urethral discharge. Cystitis tend to be more prominent compared to those of urethritis. In both men and women, symptoms are of sudden onset (Engel and Schaeffer, 1998). They include dysuria, increased frequency, urgency, suprapubic pain, back pain, small volumes, concentrated appearance, and hematuria. If the patient is febrile, this could be a sign of bacteremia and impending sepsis. These symptoms may not be present if the patient has an indwelling catheter. Symptoms of pyelonephritis include flank pain, nausea and vomiting, costovertebral angle tenderness, fever, and, rarely, a palpable and tender kidney. Hematuria and pyuria are frequently encountered (Pewitt and Schaeffer, 1997).

2.2.5 Epidemiology

Infections caused by *P. mirabilis* are commonly caused by infected medical equipment including catheters, nebulizers (responsible for inhalation), and examination gloves (responsible for wound infections). The length of catheterization is directly related to incidence of infection. Each day of catheterization gives an infection rate of (3-5%). UTIs caused by *P. mirabilis* also occur commonly in sexually active women and men, especially those engaging in unprotected intercourse. Younger women are at greater risk than younger men; however, older men are at greater risk than older women due to the occurrence of prostate disease (foxman, 2010).

2.3 Laboratory Diagnosis

2.3.1 Specimen

Most urine specimens are obtained from adult patients via the clean catch midstream technique. Midstream urine is in most cases contaminated with the flora of the anterior urethra, which often corresponds to the pathogen spectrum of urinary tract infections. Bacterial counts must be determined if “contamination “is to be effectively differentiated from “infection.” At counts in morning urine of ($>10^5$/ml) an infection is significant bacterureia. Lower counts may also be diagnostically significant in urethrocystitis.The dipstick method, which can be used in any medical practice, is a simple way of estimating the bacterial count: a stick coated with nutrient medium is immersed in the midstream urine, then incubated. The colony count is then estimated by comparing the results with standardized images (Fritz *et al*., 2005).

Catheterizing the urinary bladder solely for diagnostic purposes is inadvisable due to the potential for iatrogenic infection. Uncontaminated bladder urine is obtainable only by means of a suprapubic bladder puncture (Fritz *et al*., 2005).

2.3.2 Microscopy

P. mirabilis are gram negative polymorphic rods, actively motile, non sporing and non-capsulated(Cheesbrough, 2007).

2.3.3 Culture Technique

P. mirabilis when cultured in Blood agar aerobically, it has a characteristic fishy odor and produce swarming due to active motility. To preventing *Proteus* swarming on blood agar by adding chemicals, drying of agar plates, using alcohol treated plates, or increasing the concentration of agar (Cheesbrough, 2007).

When cultured in MacConkey, CLED and XLD media the *Proteus* produces individual non-lactose fermenting colonies after overnight incubation at (35–37 ºC). Swarming is prevented on MacConkey agar and XLD agar because these media contain bile salts. Swarming is inhibited on CLED agar because it is electrolyte deficient (Cheesbrough, 2007).

2.3.4Polymerase Chain Reaction (PCR)

The technique PCR was used in investigating some of the genes responsible for the virulence factored in *P.mirabilis* through the use of pieces of the DNA with limited number of oligonucleotide which act as primers specialized for virulence genes in *P.mirabilis,* and it include *ureC* gene which is responsible for the production urease enzyme which is regarded as a diagnostic feature of the bacteria *of P.mirabilis* and *hpmA* gene which is responsible for producing hemolysin is considered as important virulence factor for *P.mirabilis* (Hashim*et al*., 2016).

2.4Treatment and Control

The treatment of UTIs differs according to the age of the patient, sex, underlying disease, infectious agent and whether there is lower or upper urinary tract involvement. Ciprofloxacin is a recommended drug for the treatment of UTIs (Endimiani *et al.,* 2005). Though wild-type strains of

P. mirabilis are usually susceptible to fluoroquinolones (Endimiani *et al.,* 2005; Hernandez *et al*., 2000).

P. mirabilis is intrinsically resistant to tetracycline and nitrofurantoin with intermediate resistance to chloramphenicol. *Proteus* species isolated from catheter UTI are fairly resistant to amoxicillin and trimethoprim, and an increasing number of isolates multi-drug resistance against cephalosporins, aminoglycosides, fluoroquinolones, amoxicillin clavulonate and cotrimoxazole (Wazait *et al*., 2003; Cohen *et al*., 2010).

2.4.1 Ciprofloxacin

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluroquinolone drug class. It is a second generation fluoroquinolone antibacterial (Yaseen *et al*., 2003).

Ciprofloxacin is one of the broad-spectrum antibiotic active against both gram positive and gram negative bacteria. It stops bacterial infections by interfering with the enzymes that cause DNA to rewind after being copied, which works by inhibiting DNA gyrase, topoisomerase IV and a type II topoisomerase (Drlica and Zhao, 1997).

2.4.1.1 Mechanism of Action

Fluoroquinolones and earlier quinolones are novel among antimicrobial agents in clinical use because they directly inhibit DNA synthesis. Inhibition appears to occur by interaction of the drug with complexes composed of DNA and either of the two target enzymes, DNA gyrase and topoisomerase IV. These enzymes are structurally related to each other, both being tetrameric with pairs of two different subunits. The *GyrA* and *GyrB* subunits of DNA gyrase are respectively homologous with the *ParC* and *ParE* subunits of topoisomerase IV. Both enzymes are type 2 topoisomerases, which act by breaking both strands of a segment of DNA, passing another segment through the break, and then resealing the break. For DNA gyrase, this topoisomerization reaction results in

removal of DNA supercoils, thus affecting the negative supercoiling of DNA necessary to initiate DNA replication and removes positive supercoils that accumulate before an advancing replication fork. For topoisomerase IV, the topoisomerization reaction results in separation of the interlocking of daughter DNA strands that develop during replication; this facilitates the segregation of daughter DNA molecules in to daughter cells. In both cases, fluoroquinolones appear to trap the enzyme on DNA during the topoisomerization reaction, forming a physical barrier to the movement of the replication fork (Hiasa *et al*.,1996), RNA polymerase, and DNA helicase. The collision of the replication fork with these trapped complexes triggers other poorly defined events within the cell that ultimately result in cell death (Willmott*et al.,*1994; Shea andHiasa*,*1999).

2.4.1.2 Mechanisms of Resistance

The mechanisms of fluoroquinolone resistance include one or two of the three main mechanistic categories, alterations in the drug target, and alterations in the permeation of the drug to reach its target (Wetzstein*et al.,* 1997).The primary mechanisms of resistance to fluoroquinolones are mutations that result in alteration of the target proteins, DNA gyrase (encoded by *GyrA* and *GyrB*) and topoisomerase IV (encoded by *ParC* and *ParE*), and decreased intracellular drug accumulation due to drug efflux or changes in OMPs. In several species of *Enterobacteriaceae*, decreased susceptibility or resistance to fluoroquinolones is associated with specific point mutations in *GyrA*. Additional mutations in the gyrase or topoisomerase IV genes contribute to higher levels of resistance (Weigel*et al*., 2002).

2.5 Prevention and Control

P. mirabilis is part of the normal flora of the gastrointestinal tract, and as a result the bacteria enters the urinary tract or infects medical equipment by the fecal route. Consequently, prevention includes good sanitation and

hygiene, including proper sterilization of medical equipment. Recurrent infection in otherwise healthy woman can be prevented by regularly emptying the bladder this washes bacteria out of the urinary tract and is particularly important following intercourse. The prophylactic use of antibiotic resistant strains, which subsequently cause infections that are more difficult to treat (Cedric *et al*., 2006).

Infection in catheterized patients is very common, but can be reduced by good catheter care procedures. Catheterization should be avoided if possible or kept to minimum duration (Cedric *et al*., 2006).

P. mirabilis infections can be treated with broad-spectrum ciprofloxacin penicillins or cephalosporins except in severe cases. It is not susceptible to nitrofurantoin or tetracycline and has experienced increasing drug resistance of ampicillin, trimethoprim, and ciprofloxin. In cases with severe stone formation, surgery is necessary to remove the blockage (Sabbuba*et al*., 2003).

2.6 Genome Sequencing of *Proteus mirabilis*

The gram-negative enteric bacterium *P. mirabilis* is one causes of UTIs in individuals with long term indwelling catheters or with complicated urinary tracts. *P. mirabilis* bacteriuria may lead to acute pyelonephritis, fever, and bacteremia. Most notoriously, this pathogen uses urease to catalyze the formation of kidneys and bladder stones or to encrust or obstruct indwelling urinary catheters. The genome is (4.063 Mb) long and has a G_C content of (38.88%). There is a single plasmid consisting of (36,289) nucleotides. Annotation of the genome identified (3,685) coding sequences and seven rRNA loci. Analysis of the sequence confirmed the presence of previously identified virulence determinants, as well as a contiguous (54-kb) flagellar regulon and (17) types of fimbriae. Genes encoding a potential type III secretion system were identified on a Low-G_C-content genomic island containing (24) intact genes that appear to

encode all components necessary to assemble a type III secretion system needle complex. In addition, the *P. mirabilis* HI4320 genome possesses four tandem copies of the *ZapE* metalloprotease gene, genes encoding six putative autotransporters, an extension of the ATF fimbrial operon to six genes, including an *mrpJ* homolog and genes encoding at least five iron uptake mechanisms, two potential type IV secretion systems, and (16) two-component regulators (Melanie *et al*.,2008).

2.6.1 DNA Gyrase

DNA gyrase is an enzyme found only in bacteria. This enzyme uses the energy of ATP hydrolysis to introduce negative supercoils into DNA (Corbett *et al*., 2004). Negative DNA supercoiling is essential for chromosome condensation, relieving torsional strain during replication, and promoting local melting for vital processes such as transcript initiation by RNA polymerase (Wang, 2002). DNA gyrase is an excellent target for quinolones because it is not present in eukaryotic cells and is essential for bacterial growth. This enzyme comprises two subunits, A (97 kDa) and B (90 kDa), which form an A2 B2 tetramer (Wang, 2002).

2.6.1.1*GyrA*Gene

The (97 kDa) A subunit is composed of a (59 kDa) (*GyrA*59) N-terminal domain (NTD) and a (38 kDa) C-terminal domain (*GyrA*-CTD) and contains the functional parts that are involved in DNA binding (Cabral *et al.,* 1997). It contains a catabolite-activator-protein (CAP) like domain which includes the known DNA binding helix-turn-helix (HTH) motif (Cabral *et al.,*1997; Fass *et al.,* 1998). The CAP-like structure element contains the active site tyrosine residues (Tyr122). These are crucial for the breakage and religation of the DNA (Cabral *et al.,* 1997). The structure of *GyrA*-CTD adopts a spiral circular-shaped-pinwheel fold that plays an important role in DNA wrapping (Costenaro *et al*., 2005). It is assumed to contribute to the unique ability of gyrase amongst the

topoisomerases to introduce negative supercoils into positively supercoiled and relaxed DNA (Corbett *et al*., 2004). Although the spatial orientation of (*GyrA*59) and *GyrA*-CTD has been proposed on the basis of SAXS, a high resolution structural model of the complete *GyrA* subunit is still missing (Costenaro *et al*., 2005).

2.6.1.2 *GyrB* Gene

The B-subunits amount to (90 kDa) each and are responsible for ATP binding, its hydrolysis and support DNA binding (Grossmann *et al*., 2007). The *GyrB* consists of three domains of which the (43 kDa NTD) harbors the ATPase activity (Brino *et al*., 2000). The (47 kDa CTD) of *GyrB* consists of a toprim and a tail domain and contributes to the binding of DNA via interaction with the *GyrA* subunit (Kampranis and Maxwell, 1998).

2.6.2 Topoisomerase IV

Topoisomerase IV is a homologue of DNA gyrase, comprising four subunits, two of C and two of E, encoded by the *parC* and *parE* genes, respectively. The topoisomerase IV locus was described in 1990. However, a number of quinolone resistance markers had already been described and mapped to this locus. The reaction mechanism of topoisomerase IV is similar to that of gyrase but topoisomerase IV binds to DNA crossovers rather than wrapping DNA. Topoisomerase IV is primarily involved in decatenation, the unlinking of replicated daughter chromosomes (Peter, 2003).

2.6.2.1*ParC* Gene

The *ParC* subunit is (752) amino acids long and is positioned in a manner that allows it to act favorably with certain DNA geometries, enabling it to serve as a control of substrate specificity (Champoux, 2001). The *ParC* C-terminal domain of active topoisomerase IV is thought to contribute heavily to the unique functions of the enzyme in contrast to gyrase

(Corbett *et al.,* 2005). When the activity of topoisomerase enzymes lacking the *ParC* C-terminal domain was tested, they unwound both negative and positive supercoils with similar efficiency. This finding implies that the C-terminal domain of this subunit plays a crucial role in topoisomerase IV substrate specificity. Removing the C-terminal domain of *ParC* also influenced the decatenation activity of topoisomerase IV (Corbett *et al.,* 2005).

ParC are the subunits responsible for DNA binding and the cleavage and religation reaction(Levine *et al*., 1998).

2.6.2.2 *ParE* Gene

The heterotetramer of topoisomerase IV also includes two subunits of *ParE*, a (630) amino acid domain, along with the two subunits of *ParC*. The *ParE* subunit is important in regulating the enzyme function. Only when the enzyme is bound to positive supercoils can the *ParE* subunits interact to facilitate unwinding (Crisona and Cozzarelli, 2006). Both the *ParC* and *ParE* subunits are necessary for functional topoisomerase activity (Champoux, 2001). *ParE* are responsible for ATP binding and hydrolysis (Levine *et al*., 1998).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Approach

The study was a qualitative study, aimed to highlight the importance of using conventional and molecular techniques in the detection of different mutations within the genome of *Proteus mirabilis* isolates.

3.1. 1 Study Design

A prospective hospital based study was conducted by collection of urine samples from patients with clinical manifestations of UTI during the period from June 2016 to May 2017.

3.1. 2 Study Area

Different Hospitals located in Khartoum State were included in the study which include: Military Hospital, East Nile Hospital, Soba University Hospital, Khartoum Bahri Teaching Hospital, Ahmed Gasim Hospital, Omdurman teaching Hospital and Ribat university Hospital.

3.1.3 Study Population

A total of (3895) specimens were collected from patients with symptoms of UTIs from different hospitals in Khartoum State.

3.1.4 Ethical Clearance

Proposal of this study was submitted to the Federal Ministry of Health as well as the Colleague of Medical Laboratory Sciences in Shendi University for ethical approval.

3.1.5 Data Collection

Data were collected by using a standard data questionnaire consisting of basic demographic data. Additional information also included social status, history of previous UTI, and history of using antibiotics (Appendix I).

3.2 Diagnostic Approach

3.2.1 Sample Collection

Patients were asked to clean their external genitalia with disinfectant and collect midstream urine in sterilized cap. Samples were kept in ice bag and directly transported to microbiological laboratory.

3.2.2 Media and Culture Conditions

Urine samples were cultured on CLED agar, blood agar, MacConkey agar and Nutrient agar medium and incubated over night at (37°C). Significant growth was evaluated as ($\geq 10^5$) colony-forming units CFU/mL of midstream urine, then Gram stain, biochemical test (Oxidase test, Kligler Iron Agar, Tryptophan Peptone Water, Semisolid Media, Simmon's Citrate Agar, Christensen's Urea Agar) finally sensitivity test were conducted in Muller Hinton agar (Appendix II).

3.2.2.1 Cystine Lactose Electrolyte Deficient CLED Agar

All isolates were inoculated onto Cystine Lactose Electrolyte Deficient CLED Agar (HIMEDIA, India) and plates were incubated at (37°C) for (24–48 hrs).

3.2.2.1 MacConkey Agar

All isolates were inoculated onto MacConkey agar (Oxoid Media, UK) and plates were incubated at (37°C) for (24–48 hrs.).

3.2.2.2 Blood Agar

All isolates were inoculated onto blood agar (HIMEDIA, India) and plates were incubated at (37°C) for (24–48 hrs.).

3.2.2.3 Nutrient Agar

All isolates were subcultured onto nutrient agar medium (HIMEDIA, India), and plates were incubated at (37°C) for (24–48 hrs.) in order to purify the isolated bacteria from each patient urine specimen.

3.2.3 Gram's Stain

Gram stain was essential technique for initial identification of bacterial isolates. The procedure was carried out according to (Cheesbrough, 2007) as follows; smear was prepared from overnight culture on a clean and dry slide. The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunsen burner then allowed to cool before staining. Crystal violet stain was added to smear for (30–60 seconds), and then washed by tap water. Lugol's iodine was added for (30-60 minutes) then washed by tap water and decolorized rapidly (few seconds) with acetone alcohol and washed immediately by tap water. Finally, the smear was covered with Safranin stain for (2 minutes) and washed by tap water. The back of slide was wiped clean and placed in a draining rack for smear to air dry. Drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X (Cheesbrough, 2007).

3.2.4 Biochemical Tests

3.2.4.1 Oxidase Test

The oxidase test is for the presence of cytochrome oxidase. Using a piece of stick or glass rod (not an oxidized wire loop), removed colony of the test organism and smeared it on filter paper soaked with a few drops of oxidase reagent, Looked for the development of a blue-purple colour within a few seconds (Cheesbrough, 2007).

3.2.4.2 Kligler Iron Agar(KIA)

Kligler Iron Agar is used for the differentiation of microorganisms on the basis of dextrose and lactose fermentation and hydrogen sulfide production. Using sterile straight loop The colonies were touched and inoculated on Kligler iron agar (HIMEDIA, India) and then incubated at (37 ºC) overnight incubation(Cheesbrough, 2007).

3.2.4.3 Urease Test

Testing for urease enzyme activity is important in differentiating enterobacteria. *Proteus* strains are strong urease producers. The test organism is cultured in a medium which contains urea and the indicator phenol red (HIMEDIA, India), and then incubated at (37 ºC) overnight incubation (Cheesbrough, 2007).

3.2.4.4 Simmon's Citrate Agar

This test is one of several techniques used occasionally to assist in the identification of enterobacteria. Using sterile straight loop, the colonies were touched and inoculated the butt of Simmon's citrate agar (HIMEDIA, India) and then incubated at (37 ºC) overnight incubation (Cheesbrough, 2007).

3.2.4.5 Indole Test

Testing for indole production is important in the identification of enterobacteria. Most bacteria break down the amino acid tryptophan with the release of indole, the tested organism inoculated to tube containing (3 ml) of sterile tryptone water (HIMEDIA, India), and then incubated at (37 ºC) overnight incubation then interpreted after adding kovac's reagent to tryptophan peptone water medium (Cheesbrough, 2007).

3.2.4.6 Motility Test

Testing for motility and swarming is important in the identification of enterobacteria**.** Using sterile straight loop, the colonies were touched and inoculated the butt of semisolid media (HIMEDIA, India), and then incubated at (37 ºC) overnight incubation(Cheesbrough, 2007).

3.2.5 Antimicrobial Susceptibility Testing

Susceptibility pattern was done by modified Kirby-Bauer method. All the isolated organisms were put into appropriate media for antibiotic susceptibility test by Kirby-Bauer disc diffusion technique. Disc diffusion tests were performed and interpreted according to the recommendations

of the Clinical and Laboratory Standards Institute (CLSI, 2010). All tests were performed on Muller-Hinton agar plates (pH 7.2-7.4). The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to (0.5) McFarland standards. The swab stick was then taking out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at (37 ºC) for (24 hrs). On the next day, plates were read by taking measurement of zone of inhibition. Inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open. They were held a few inches above a black, nonreflecting background and illuminated with reflected light. Results were recorded and graded as resistant (R),intermediate (I) and sensitive (S), according to the reference zone of inhibition of particular antibiotic (NCCLS, 2001).

3.3 Molecular Approach

3.3.1 Polymerase Chain Reaction

3.3.1.1 DNA Extraction for Polymerase Chain Reaction

Genomic DNA templates for PCR amplification were gained from overnight growth of bacterial isolates on nutrient agar suspended in (500 µL) of sterile deionized water, and boiled for (10 minutes). After centrifugation of the boiled samples at (14000 g) for (10 minutes) (Sigma 1-14 Germany Microcentrifuge) (Appendix IV - Figure 34), supernatant was stored at (20°C) as a template DNA stock (Kazuki *et al*., 2014). The purity of the extracted DNA was determined by running the DNA sample on (2%) gel agarose (Sambrook *et al*., 1989).

3.3.1.2 Primer Design

Degenerate oligonucleotide primers (Table 1 and Appendix III) from conserved regions of the *GyrA*, *GyrB*, *ParC* and *ParE* genes were designed by primer3plus (www.bioinformatics.nl/primer3plus) from

Proteus mirabilis HI4320 DNA sequences in the GenBank database (NCBI) and synthesized by Macrogen (South Korea).

Table (1) Primers used for detection of virulence genes in *Proteus mirabilis* strains

Primer	primers Sequence	Product size bp
Gyr A	F 5′- AGCGACATTGCCAGAGAAAT -3′ R 5′- CACCGACTGCATCACGTTT -3′	937
Gyr B	F 5′- GGCAAAACAAGGGCGTAA-3′ R 5′- GCCCCTTCTTCAATCAGGTT-3′	822
Par C	F 5′- CAGCGTCGTATCGTCTATGC-3′ R 5′-CGGCGTAATACTTTTTCTAAGC-3′	992
Par E	F 5′- GGAAGGAGGCGATTTACTCA-3′ R 5′-GGATCAAGCGTTGTCTCACG-3′	972

3.3.1.3 Amplification of *GyrA*, *GyrB*, *ParC* and *ParE* Genes

The amplification was done using (CLASSIC K960 China thermal cycler) (Appendix IV- Figure 35). DNA amplifies was done using Maxime PCR Premix kit (*I*-Taq) (iNtRON, Korea) (Appendix V). The PCR assay was carried out in a total volume of (20 µL) of mixture containing (0.5 µL) of each of the virulence gene-specific primers (1 µL), (2 µL) of template DNA and (17 µL) of water for injection (WFI). The amplification conditions included three steps: heating at (94°C) for (5 minutes); (35 cycles) of denaturation at (94°C) for (30 seconds), annealing at (55°C) for (30 seconds), and extension at (72°C) for (30 seconds); and the final extension at (72°C) for (3 minutes) (Weigel*et al*., 2002).

3.4.1.4 Visualization of the DNA

The gel casting tray was placed into the electrophoresis system, tank flooded with 1x TBE buffer just to cover the gel surface, (5 µl) of PCR products from each samples was added to wells of electrophoreses, (5 µl) of DNA ladder (100-bp DNA ladder, iNtRON, Korea) (Appendix VI), was added to the well in each run. The gel electrophoresis apparatus was

connected to power supply (Primer, 100 V, 500 mA, UK) (Appendix IV-Figure 36). The electrophoresis was carried out at (75Volts) for (30 minutes) and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminator and photographed (Uvitec – UK) (Appendix IV-Figure 37).

3.3.2 Restriction Fragment Polymorphism(RFLP-PCR)

The PCR product was digested with *HinfI* (CutSmart^{TM} , New England Biolabs, Inc) restriction endonuclease digestion under conditions recommended by the manufacturer to detect *GyrA* (ser 83) and *ParC* (ser 81) mutations. It was carried out in a total volume of (50µL) of mixture containing 20µL DNA product, 1µL restriction enzyme, 5µL (10X) NE Buffer, 24µL of WFI (water for injection) and incubated at 37^{o}C for 5-15 min (Appendix VII). The amplified and the digestion products were resolved by electrophoresis in agarose (2%) gels and visualized by ethidium bromide staining (figure 1) (Shang *et al*., 2009)

3.3.3 Sequencing of the Target Genes and Detection of Mutations

Three product selected randomly to detect *GyrA* and *GyrB*, *ParC* and *ParE*, the sequencing done in South Korea after transport by DHL company for product to Macrogens Inc (info@macrogen.com) in both directions with the same set of primers used for the PCR by Sanger dideoxy chain termination method.

3.3.3.1 Data and Genetic analysis

The data analysis by suitable statistical tests using statistical software package (SPSS - version 20). The sequences were checked for similarity with reference genes using NCBI's BLAST (http://www.ncbi.nlm.nih.gov/blast). The sequences were translated into amino acid codon using Expasy translation tool. The protein sequences were then checked for similarity in BLAST.

CHAPTER FOUR

RESULTS

4.1 Demographic Data

A total of (3895) urine specimens were collected from patients with symptoms of UTIs from Military Hospital (809), East Nile Hospital (148), Soba University (410), Khartoum Bahri Teaching Hospital (415), Ahmed Gasim Teaching Hospital (346), Omdurman Teaching Hospital (1319) and Ribat University Hospital (448). Among the study population 2085 (53.5%) were females while 1810(46.5%) were males, among these *P. mirabilis* were identified in 120 (3.1%) patients (66 patents 55% were females and 54 patents 45% were males) as shown in (Figure 1). The difference rate in male among female statistically insignificant at P-value=0.743. Also other data were registered in the submitted questionnaire (Appendix 1).

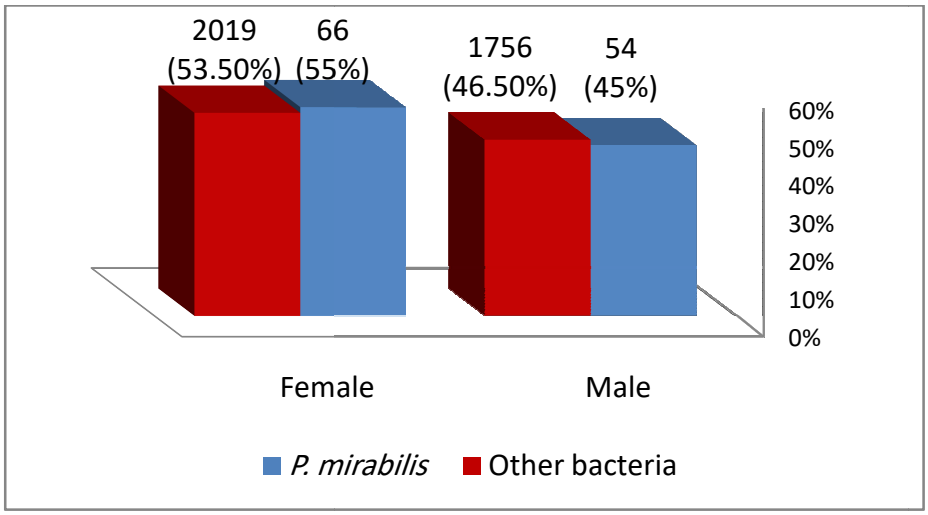


Figure (1) Relation between *Proteus mirabilis* and gender compared to other isolates (P-value=0.743)

4.1.1Enrolled Patients Versus Age Groups

Patients enrolled in the study were divided into three age groups: less than (10 yrs), (11- 49 yrs), and more than (50 yrs). The highest frequency of isolates 2087 (53.6%) was in the age group (11-49yrs),

followed by the age group of more than (50 yrs) 1663 (42.7%) while the lowest frequency of isolates 145 (3.7%) in the age group of less than (10 yrs) as shown in (Figure 2). The difference rate age group statistically significant at p-value=0.000.

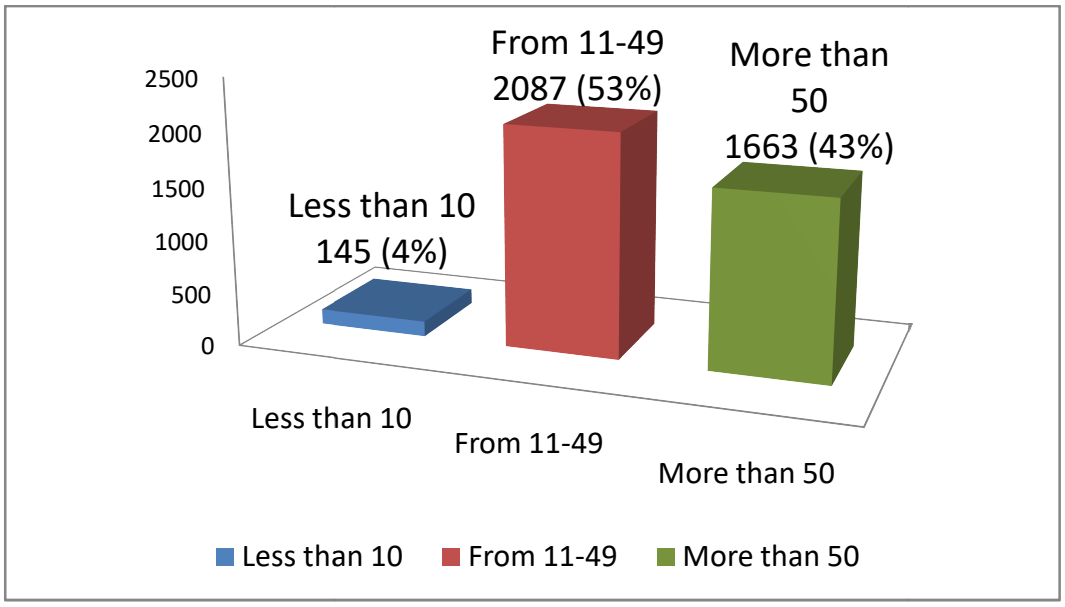


Figure (2) Frequency of the isolates according to age groups **(**p-value=0.000)

4.2 Bacteriological Findings

4.2.1 Identification Scheme

The bacterial isolates obtained in this study were identified according to their cultural characteristic, colonial morphology, Gram reaction and their biochemical properties, the total number of bacterial isolates were (3895), 3472 (89.1%) were Gram negative isolates and 423(10.9%) were Gram positive. The identification scheme confirmed that 120 (3.1%) of isolated ware belonging to the species *P. mirabilis*, 2185(56.1%) were *E. coli*, 703 (18.0%) were *K. pneumoniae* , 85 (2.2%) were *P.vulgaris*,348 (8.9%) were *Ps. aeruginosa*, 31 (0.8%) were *Citrobacter* spp., 386 (9.9%) were *E. faecalis*, 23 (0.6%) were *S.epidermidis* and 14 (0.4%) were *S. aureus* as illustrated in Figures 3,4,5,6,7,8, 9 and 10.

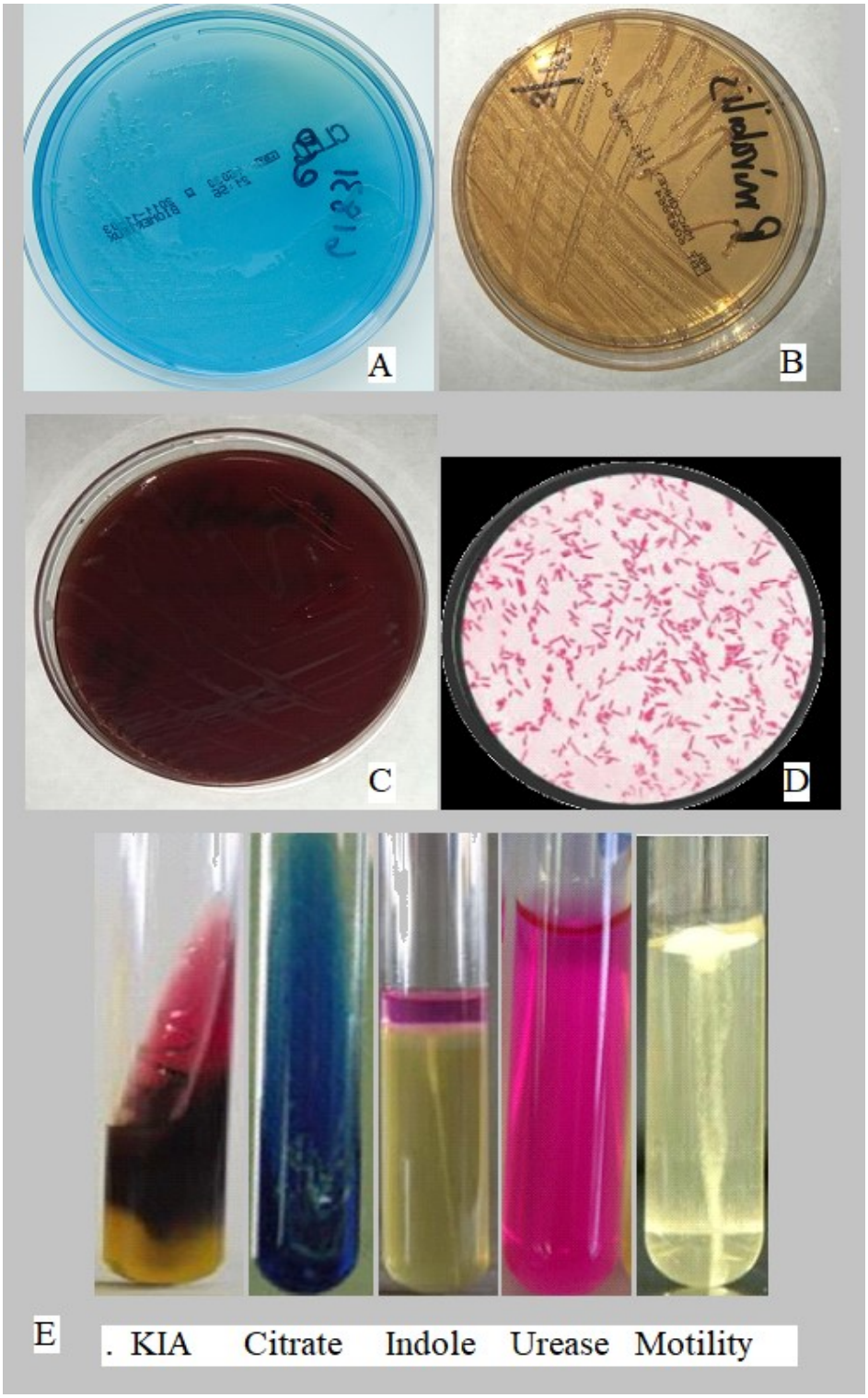


Figure (3) Identification of the *P. mirabilis* (A); Overnight growth of *P. mirabilis* on CLED media which produces blue color duo to none lactose fermenting,(B); Overnight growth of *P. mirabilis* on MacConkey media which produces yellow color duo to none lactose fermenting,(C); Overnight growth of *P. mirabilis* on blood agar medium which produced swarming, (D); *P. mirabilis* under microscope with X100 objectives, (E); Biochemical test of *P. mirabilis* (KIA none lactose fermented, glucose

fermented without gas and H_2s produced, citrate positive, Produced of indole after adding of Kovac's reagent, urease positive and highly motile bacteria)

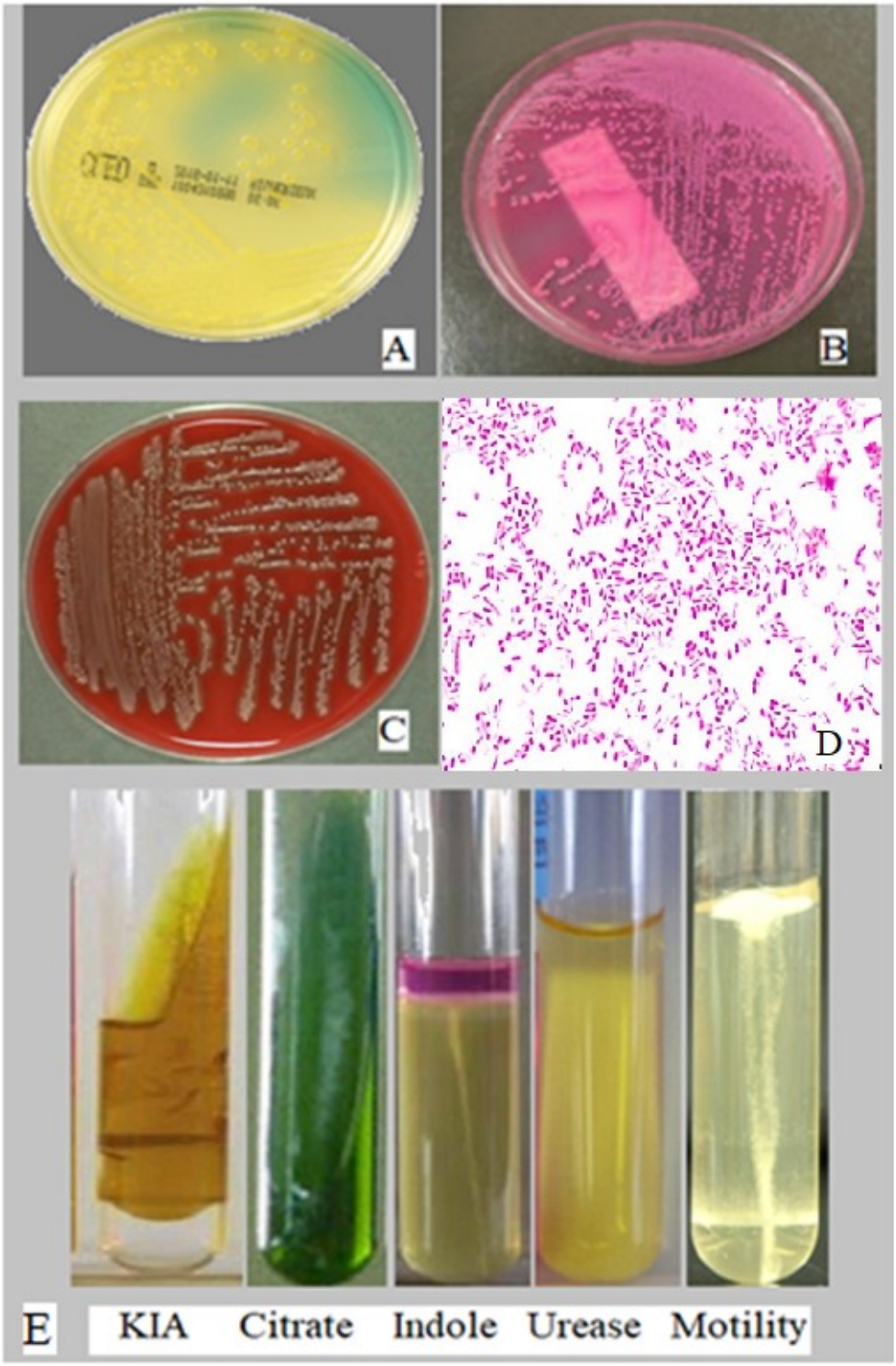


Figure (4) Identification of the *E. coli*(A); Overnight growth of *E. coli* on CLED media which produces yellow color duo to lactose fermenting, (B); Overnight growth of *E. coli* on MacConkey media which produces pink color duo to lactose fermenting,(C); Overnight growth of *E. coli*on blood agar medium, (D); *E. coli* under microscope with X100 objectives, (E); Biochemical test of *E. coli*(KIA: lactose and glucose fermented with gas produced and H2s none produced, citrate negative,

Produced of indole after adding of Kovac's reagent, urease negative and motile bacteria)

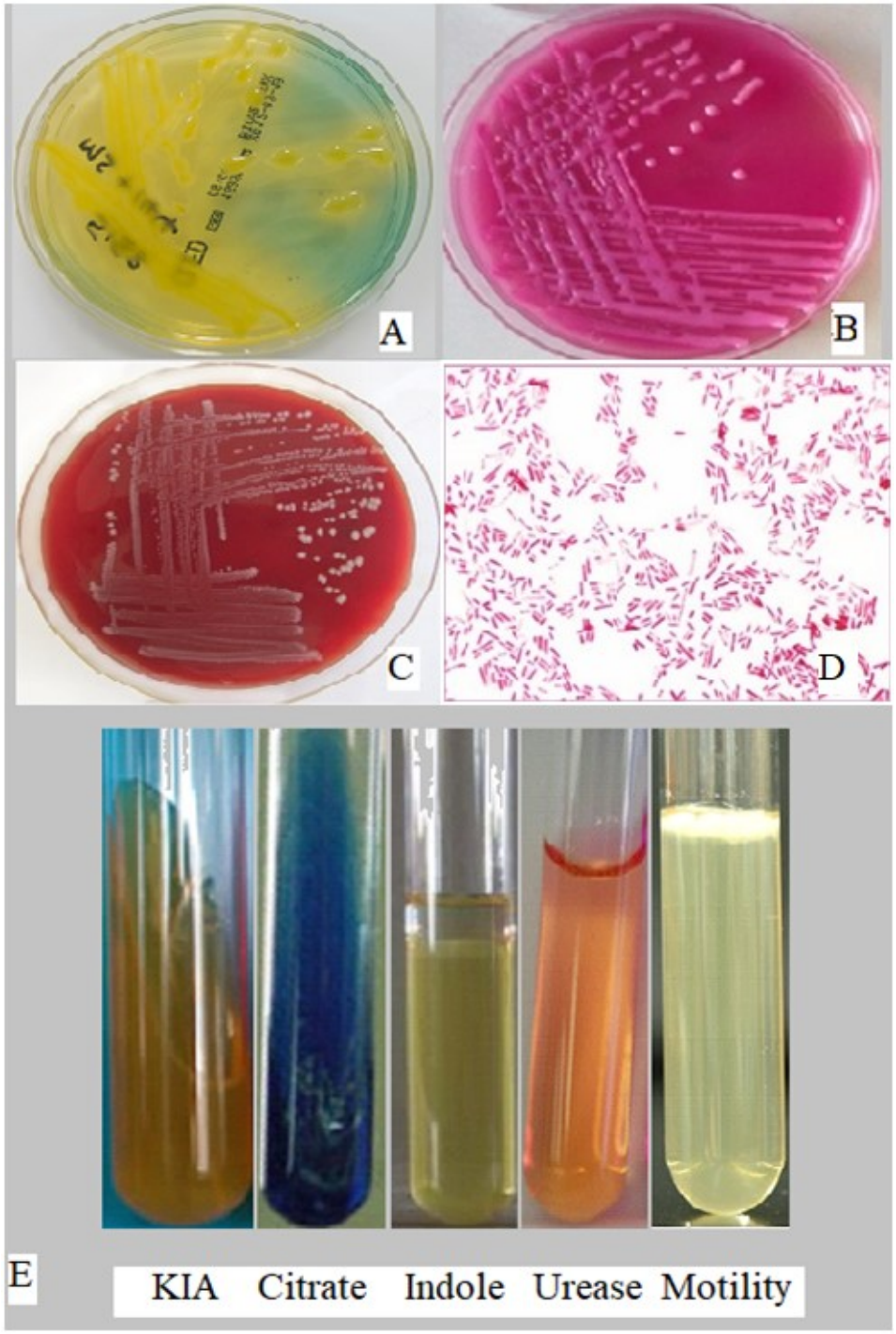


Figure (5) Identification of the *K. pneumoniae* (A); Overnight growth of *K. pneumoniae* on CLED media which produces yellow color duo to lactose fermenting,(B); Overnight growth of *K. pneumoniae* on MacConkey media which produces pink color duo to lactose fermenting,(C); Overnight growth of *K. pneumoniae* on blood agar medium, (D); *K. pneumoniae* under microscope with X100 objectives, (E); Biochemical test of *K. pneumoniae* (KIA: lactose and glucose fermented without gas and H2s produced, citrate positive, None produced of indole after adding of Kovac's reagent ,urease weakly positive and None motile bacteria)

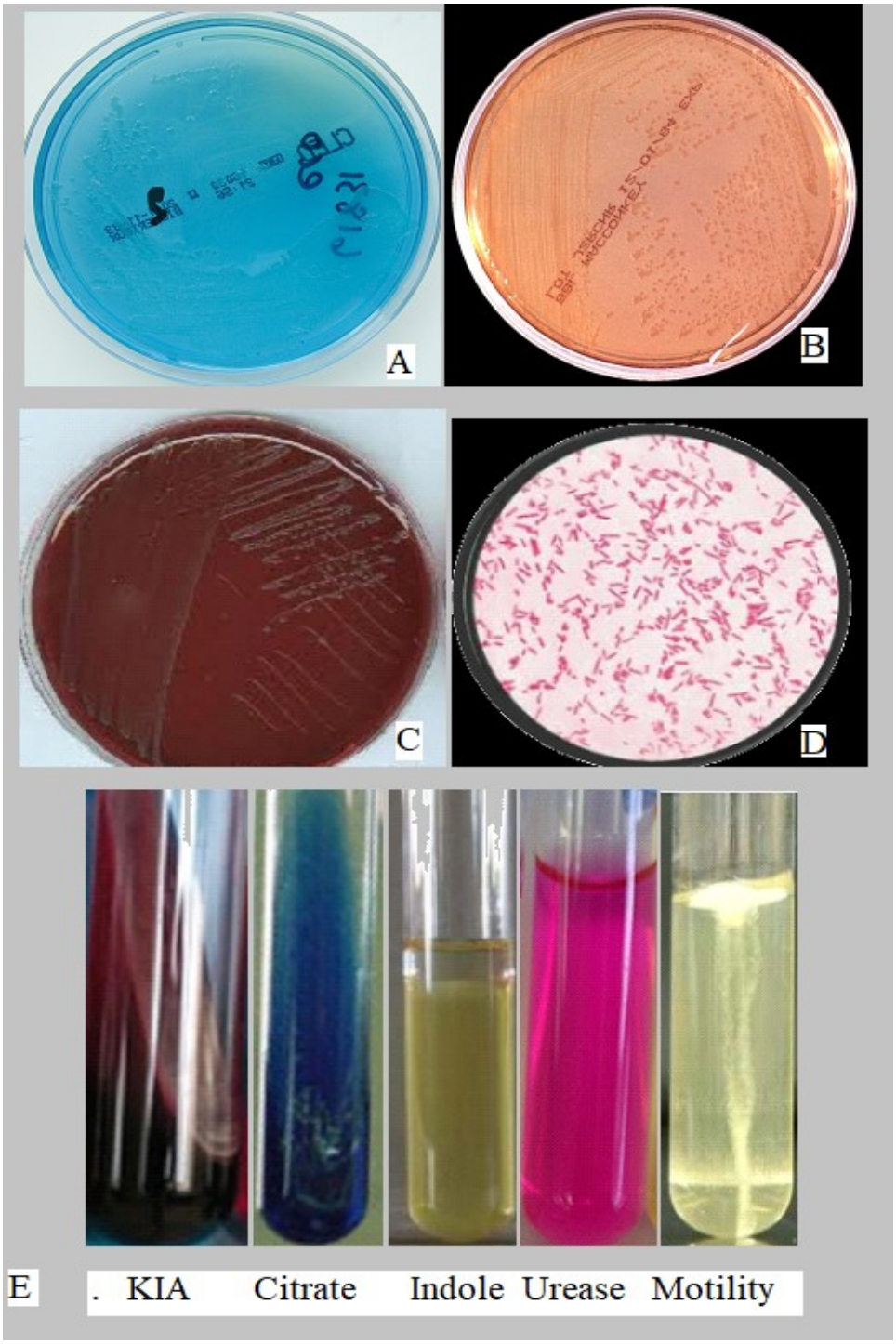


Figure (6) Identification of the *P. vulgaris* (A); Overnight growth of *P. vulgaris* on CLED media which produces blue color duo to none lactose fermenting,(B); Overnight growth of *P. vulgaris* on MacConkey media which produces yellow color duo to none lactose fermenting,(C); Overnight growth of *P. vulgaris* on blood agar medium which produced swarming, (D); *P. vulgaris* under microscope with X100 objectives, (E); Biochemical test of *P. vulgaris* (KIA none lactose fermented, glucose fermented without gas and H_2s produced, citrate positive, None produced of indole after adding of Kovac's reagent, urease positive and highly motile bacteria)

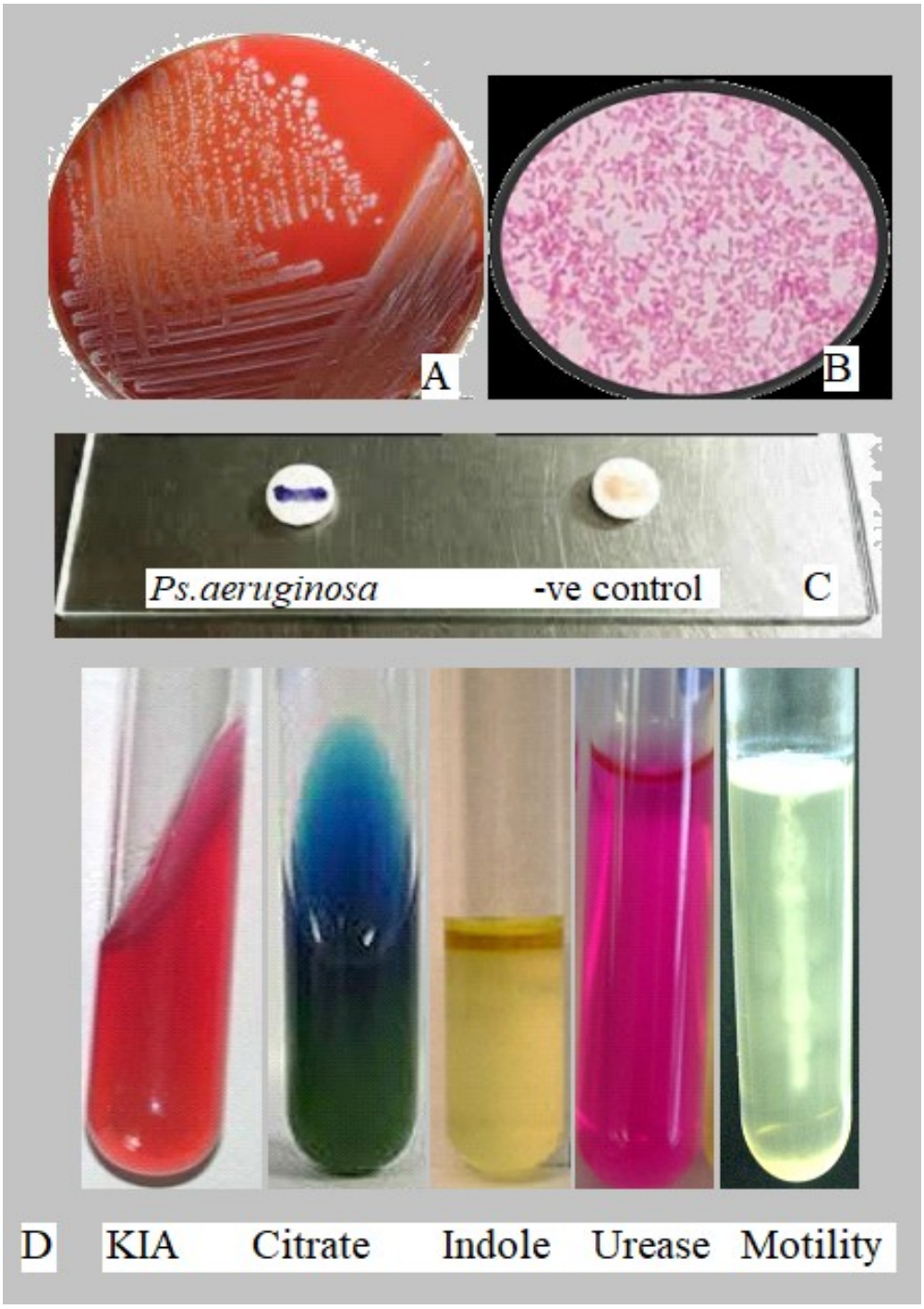
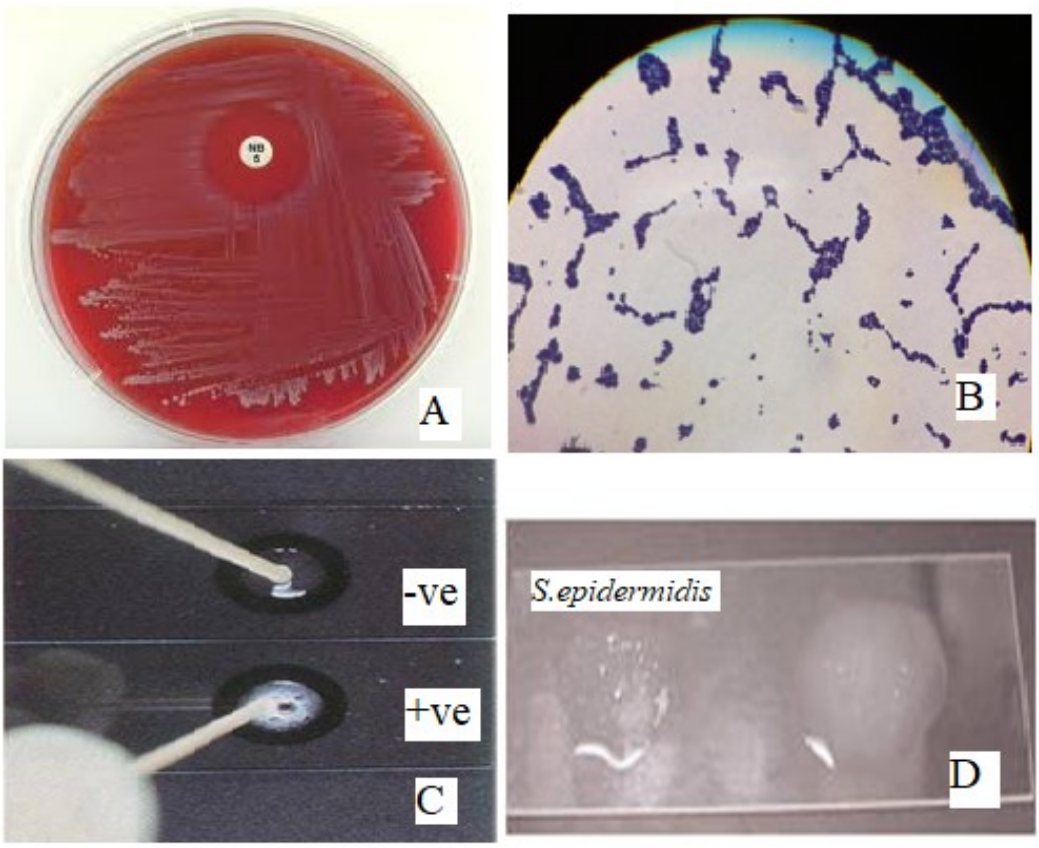


Figure (7) Identification of the *Ps. aeruginosa* (A); Overnight growth of *Ps.aeruginosa* on blood agar medium, (B); *Ps.aeruginosa* under microscope with X100 objectives,(C); Cytochrome oxidase enzyme produced by *Ps. Aeruginosa*,(D); biochemical test of *Ps.aeruginosa* (KIA none lactose and glucose fermented without gas and H_2S,citrate positive, None produced of indole after adding of Kovac's reagent, urease positive and motile bacteria)



Fugue (8) Identification of the *Streptococcus epidermidis*(A);Overnight growth of *S. epidermidis* on blood agar medium which produces grey color with novobiocin disk, (B);*S. epidermidis* under microscope with X100 objectives,(C);Oxygen bubbles formation by *S. epidermidis* (catalase test),(D);No clumping in coagulated plasma drop by *S. epidermidis* (coagulase test).

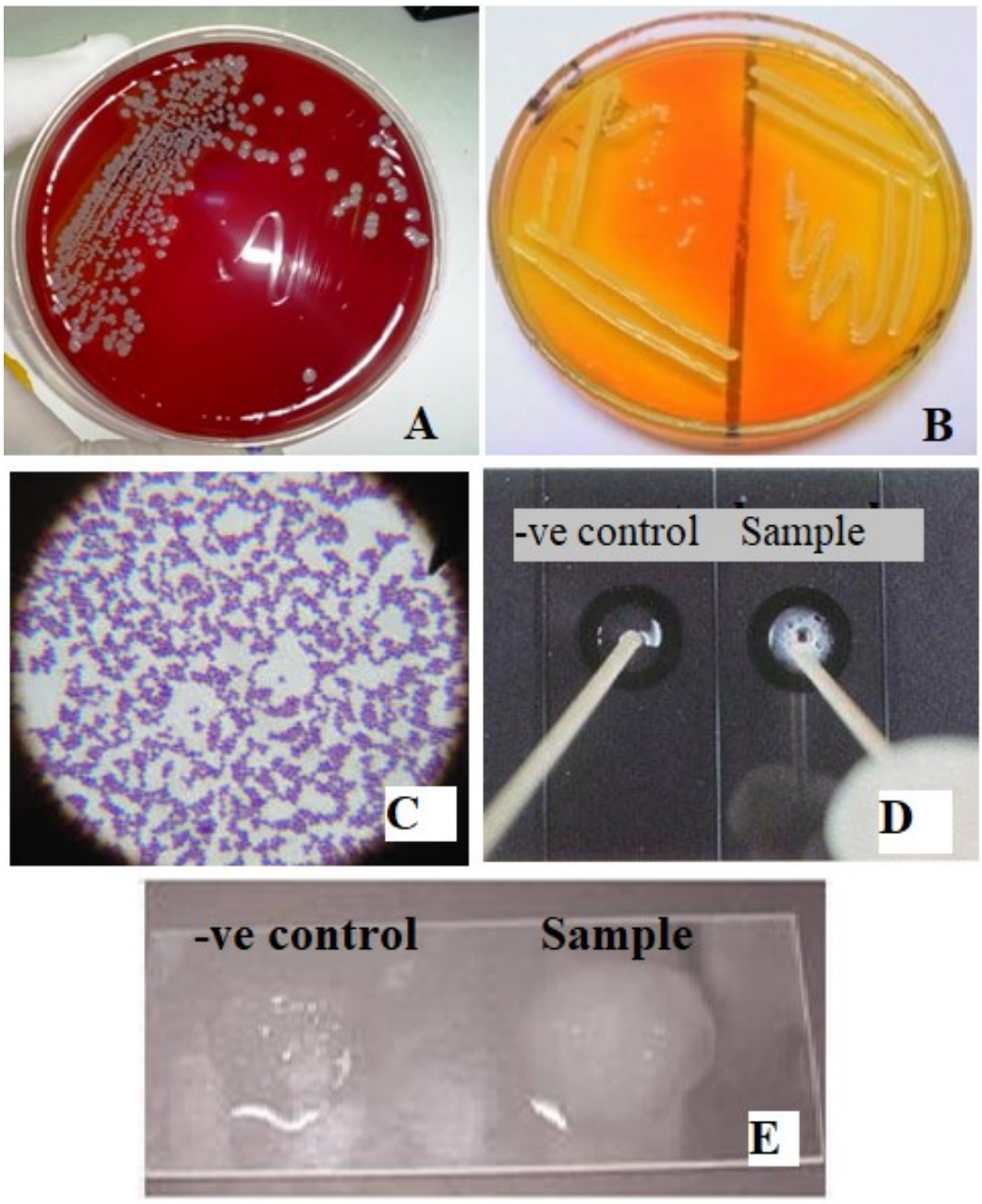


Figure (9) Identification of the *S. aureus* (A); Overnight growth of *S. aureus* on blood agar medium which produces grey color, (B); Fermentation reaction of *S. aureus* on MSA medium,(C); *S. aureus* under microscope with X100 objectives, (D); Oxygen bubbles formation by *S. aureus* (catalase test), (E); Clumping in coagulated plasma drop by *S. aureus* (coagulase test).

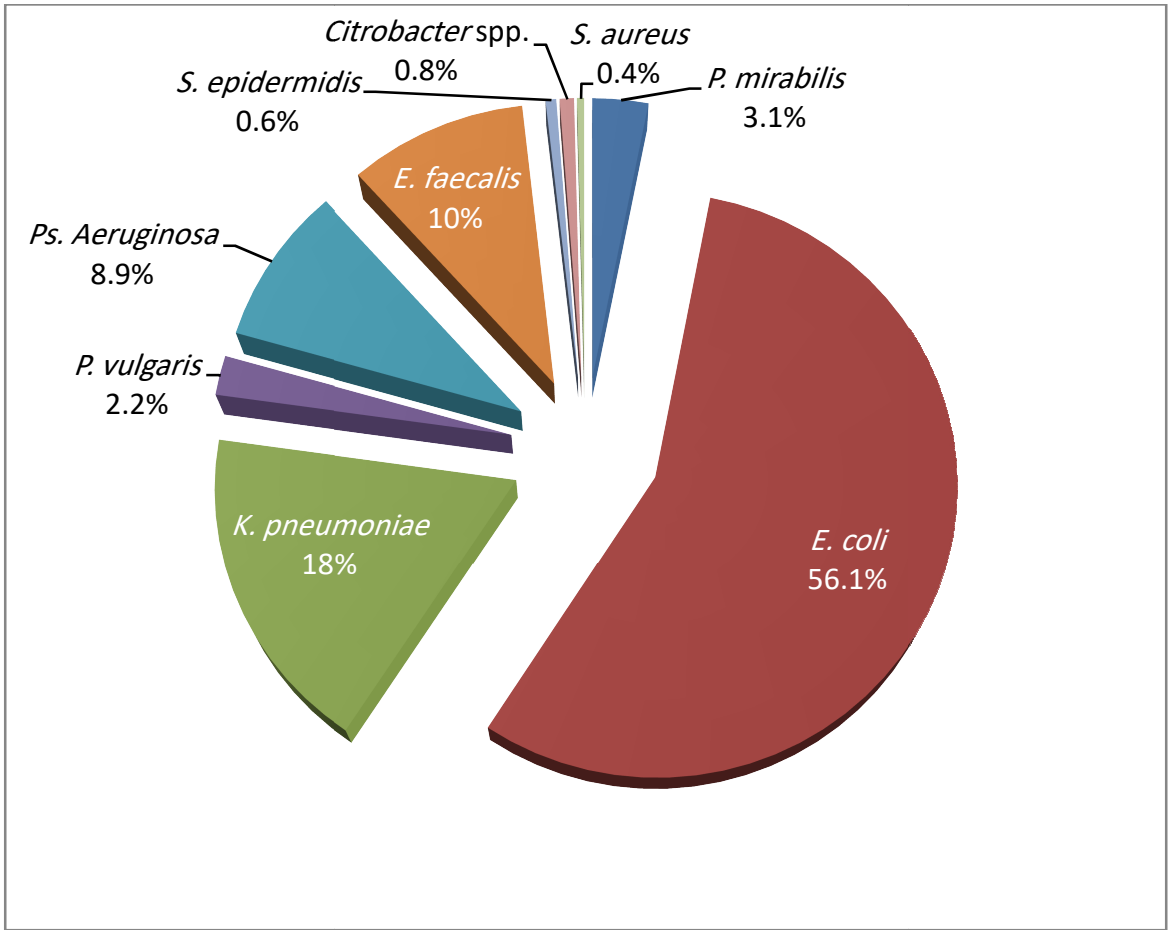


Figure (10) Frequency of the isolated bacterial species from urine samples

4.2.2 Results of Modified Kirby-Bauer Technique

The results of modified Kirby-Bauer method showed that *P. mirabilis* reflected decrease sensitivity to ciprofloxacin 84 (70%) sensitive and 36 (30%) resistant (Figure 11), while the other bacteria 995 (26.36%) Sensitive, 23 (0.61%) Intermediate and 2757 (73.03%) resistant to ciprofloxacin as shown in (Figure 12 and 13). The difference rate of sensitivity test statistically significant at p-value=0.000.

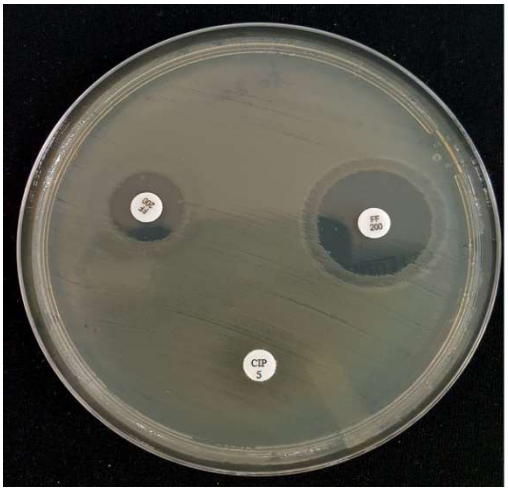


Figure (11) Susceptibility test of *P. mirabilis* to ciprofloxacin by modified Kirby-Bauer method

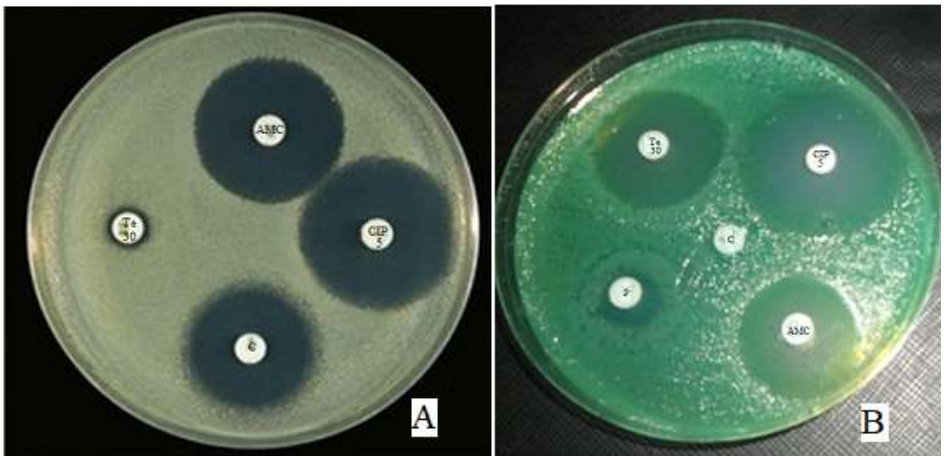


Figure (12) Susceptibility test of (A) *E. coli* (B) *Ps. aeruginosa* ciprofloxacin by modified Kirby-Bauer method

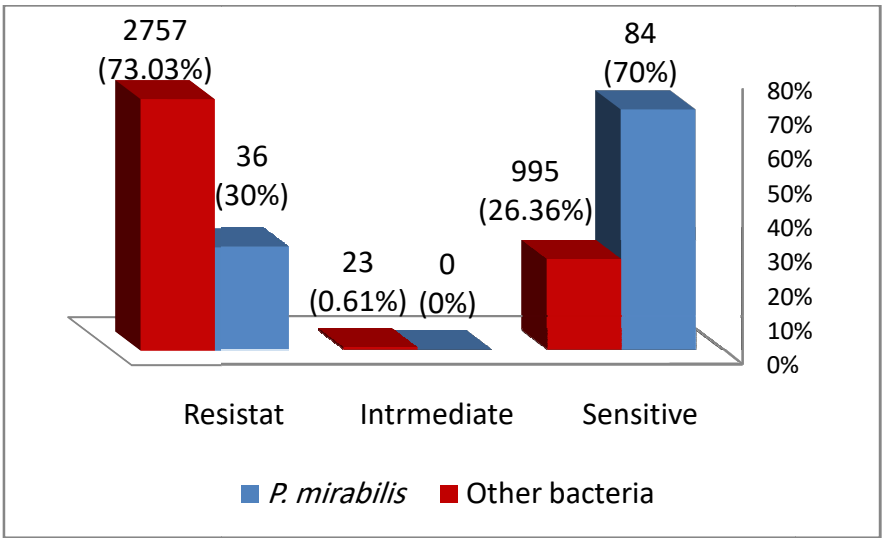


Figure (13) Sensitivity of *P. mirabilis* against ciprofloxacin comparing to other non-*Proteus* isolates (p-value=0.000)

4.3 Molecular Findings

4.3.1 Purity of the Extracted DNA Chain Reaction

P. mirabilis extracted DNA purity was detected by (2%) agarose gel, the extracted DNA was clearly seen in pure form and high amounts compared to the DNA marker which contain (40 ng) in (5µL) loading (all fragments except typical band DNA). The typical band of DNA fragments is (100 ng) (figure 14).

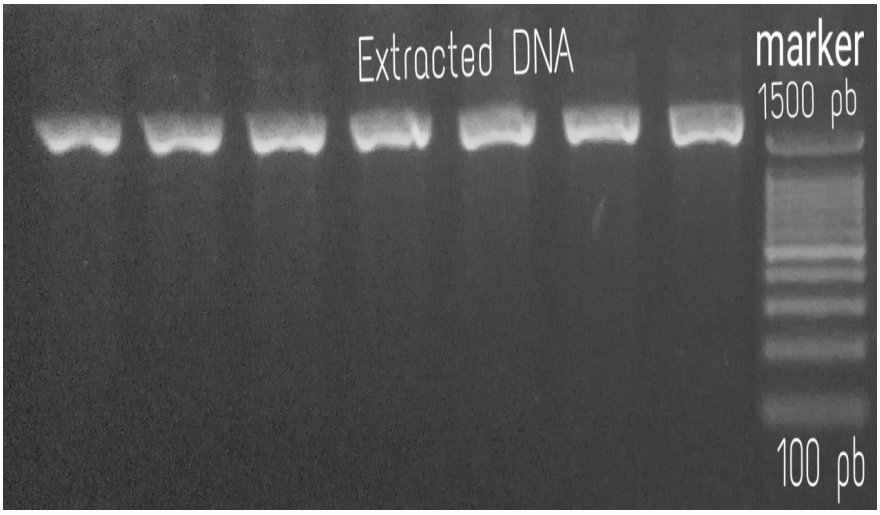


Figure (14) *P.mirabilis* extracted DNA separated by 2% agarose gel

4.3.2 PCR for the amplification of *GyrA*, *GyrB*, *ParC* and *ParE* Genes

Degenerate oligonucleotide primers from conserved regions of the *GyrA*, *GyrB*, *ParC* and *ParE* genes were designed from alignments of known DNA sequences in the GenBank database (NCBI). PCR amplified *GyrA* gene product (937 bp) which encoded (312) amino acids, *GyrB* Gene product (822) which encoded (274)P amino acids, *ParC* Gene product (992) which encoded (230) amino acid and *ParE* Gene product (972) which encoded (324) amino acids of all the isolates *P. mirabilis* on the agarose gel (Figure15).

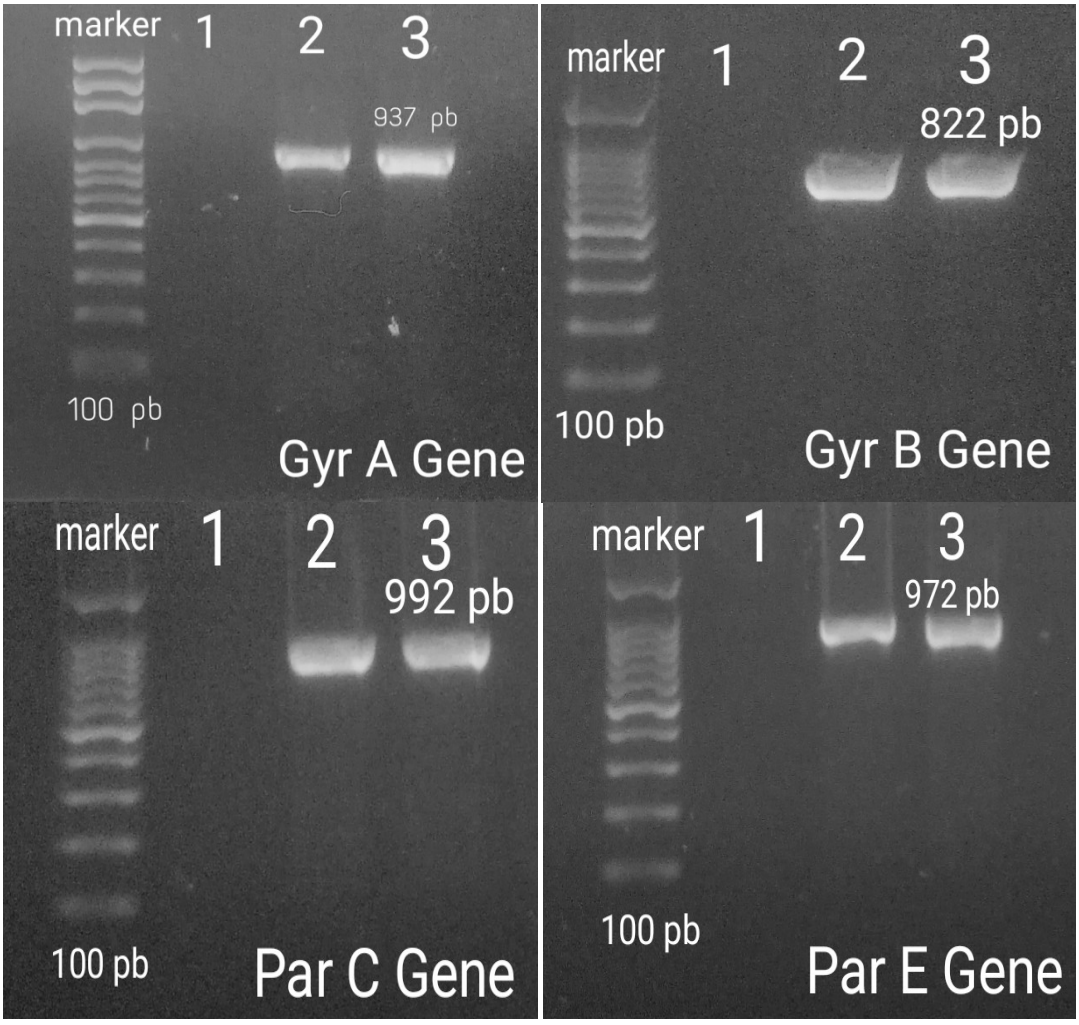


Figure (15) PCR products of *GyrA*, *GyrB*, *ParC* and *ParE* separated by 2% agarose gel : lane 1: Negative control lane 2: and 3: PCR products

4.3.3*Hinf1*Digestion to Screen mutations in *GyrA* and *ParC* Genes

Quick screening of (120) *P.mirabilis* to detect the mutation at the codon (83 ser) of the *Gyr*A gene and (84 ser) of *ParC*, was done by *Hinf1* restriction endonuclease digestion. PCR amplified *GyrA* gene product (937 bp) of all the isolates (mutant 83ser :two band 601 and 336 bp, non-mutant 83 ser: three band 601, 200 and 136 bp), when digested with the *Hinf1* restriction endonuclease enzyme gave two bands of (601 and 336 bp) and *ParC* product (992 bp) of all the isolates (mutant 84ser :two band 709 and 273 bp, non-mutant 84 ser: three band 656, 273 and 53 bp), when digested with the *Hinf1* restriction endonuclease enzyme gave two

bands of (709 and 273 bp) on the agarose gel (Figure16) all samples mutate at (serine 83) of *GyrA* and (serine 84) of *ParC*.

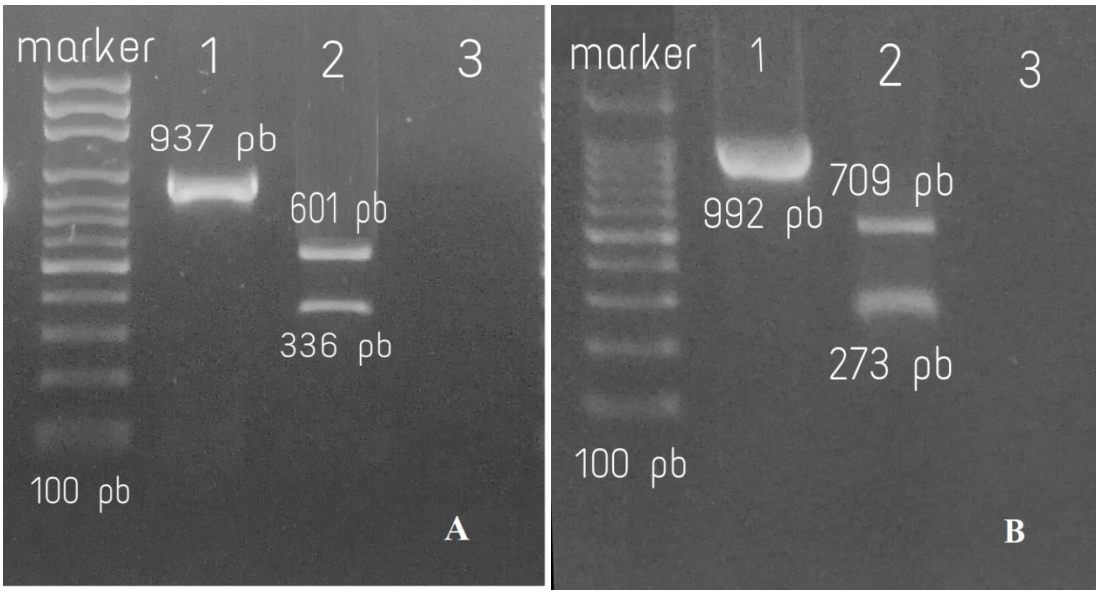


Figure (16) PCR products of *GyrA* and *ParC* were digested with *HinfI* and separated by 2% agarose gel. A (*GyrA*): lane 1: non-digested products (937 bp), lane 2: *HifI*-digested product (601 and 336 bp) lane 3: negative control and B (*ParC*): lane 1: non-digested products (992 bp), lane 2: *HifI*-digested product (709 and 273 bp) lane 3: negative control

4.3.4 Sequencing of *GyrA*, *GyrB*, *ParC* and *ParE* Genes and Detection of Mutations

Sequencing of *GyrA* QRDR of *P. mirabilis* alignment with reference *P. mirabilis* HI4320 strain in GenBank database NCBI by nucleotide blast as shown in (figure 18) revealed mutations at (codon 83) (Table 2) possessed serine to isoleucine (figure 17) substitution (G 248 T) this was observed in one strain (figure 19).

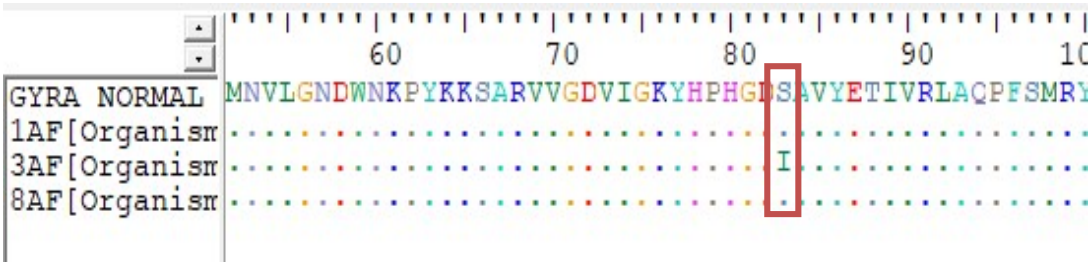


Figure (17) *GyrA* amino acid changed codon 83 serine to isoleucine (AGT- ATT). Analyses was done by BioEdit alignment editor v7.2.5

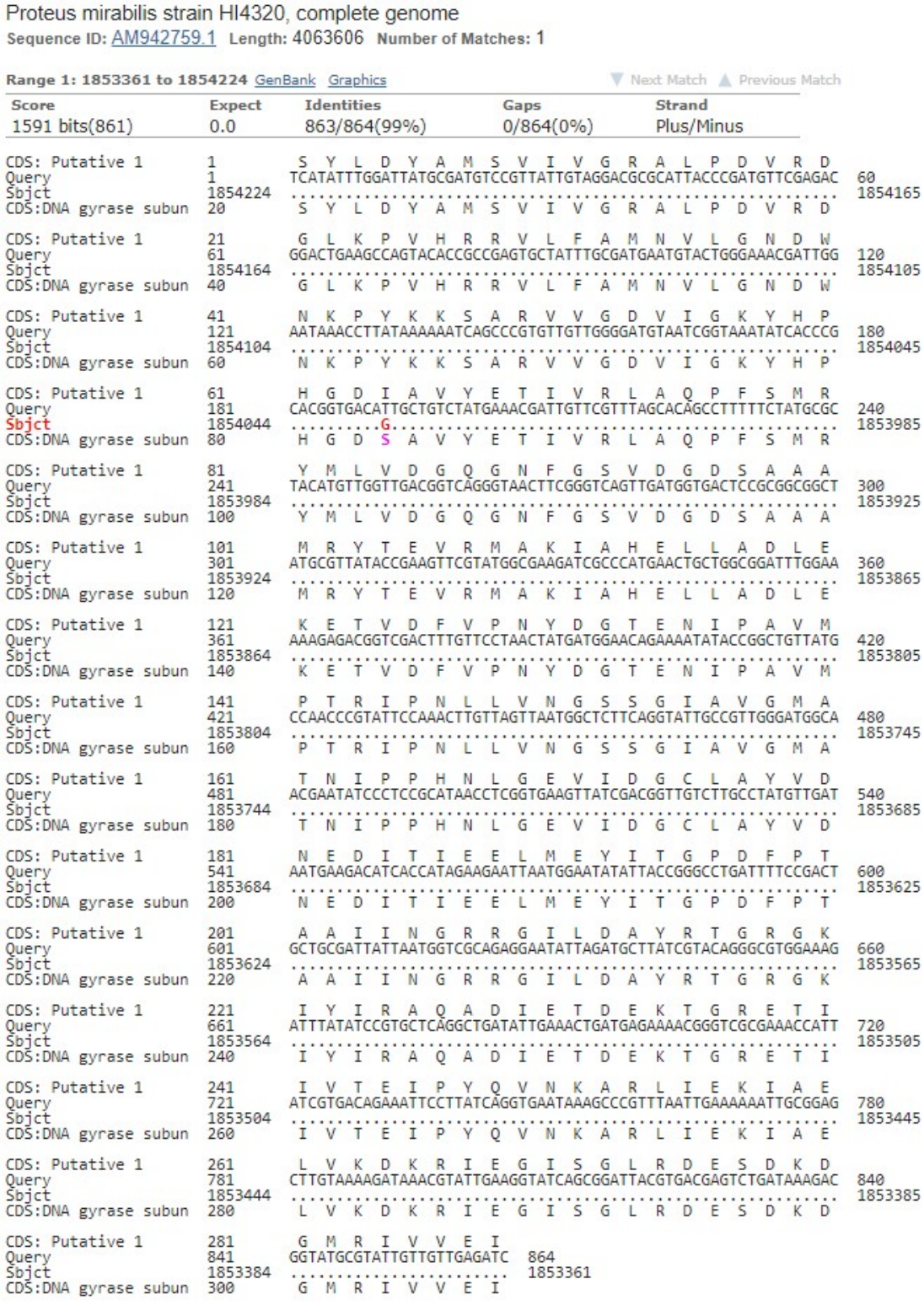
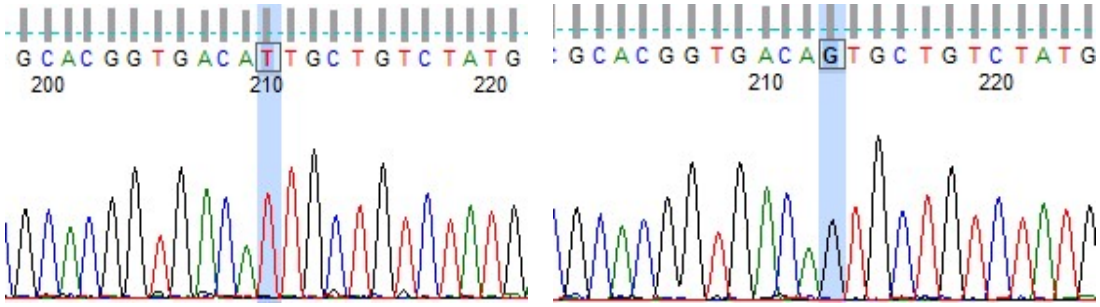


Figure (18) Alignment of *P. mirabilis GyrA* with reference *P. mirabilis* HI4320 strain



Sample 3A Wild type of *GyrA*

Figure (19) Chromatograms of Sanger DNA sequencing sample 3A of *GyrA* changed from G to T which change serine to isoleucine and wild type of *GyrA* gene

Table (2) Accession numbers, ciprofloxacin susceptibility and QRDR mutations of *Proteus mirabilis* isolates.

Sample	accession numbers	Ciprofloxacin susceptibility	Target gene	Amino acid change	
				Amino acid	Nucleotide
1A	MH310924	Resistance	*GyrA*	-	-
3A	MH310925	Resistance	*GyrA*	Ser 83 Ile	AGT-ATT
8A	MH310926	Sensitive	*GyrA*	-	-
1B	MH310921	Resistance	*GyrB*	Lus 474 Lus	TTA -TTG
				Val 585 Val	GTT -GTC
3B	MH310922	Resistance	*GyrB*	Lus 474 Lus	TTA -TTG
				Val 585 Val	GTT -GTC
8B	MH310923	Sensitive	*GyrB*	Lus 474 Lus	TTA -TTG
				His 612 His	CAC-CAT
				Asn 639 Asn	AAT AAC
1C	MH310927	Resistance	*ParC*	Ser 84 Ile	AGT-ATT
3C	MH310928	Resistance	*ParC*	Ser 84 Ile	AGT-ATT
				Pro 116 Pro	CCA CCT
8C	MH310929	Sensitive	*ParC*	His 81 His	CAC CAT
1E	MH310930	Resistance	*ParE*	-	-
3E	MH310931	Resistance	*ParE*	Ile 469 Ile	ATC -ATT
				Asp 531 Asp	GAC-GAT
8E	MH310932	Sensitive	*ParE*	Ile 469 Ile	ATC -ATT
				Asp 531 Asp	GAC-GAT
				Glu 533 Glu	GGT-GGA

Sequencing of *GyrB* QRDR of *P. mirabilis* alignment with reference *P. mirabilis* HI4320 strain in GenBank database NCBI by nucleotide blast as shown in (figure 21) revealed Silent mutations at following codons 474, 585, 612 and 639 (Table 2). Codon 474 leucine in all strains, including sensitive strain (8B) substitution (A 1422 G) figure (20), codon 585 valine in resistance strains (1B and 3B) substitution (T1 755 C) (figure 22), codon 612 histidine in sensitive strain (8B) substitution (C1836T) (figure 23) and codon 639 asparagine in sensitive strain (8B) substitution (T1917C) (figure 24).

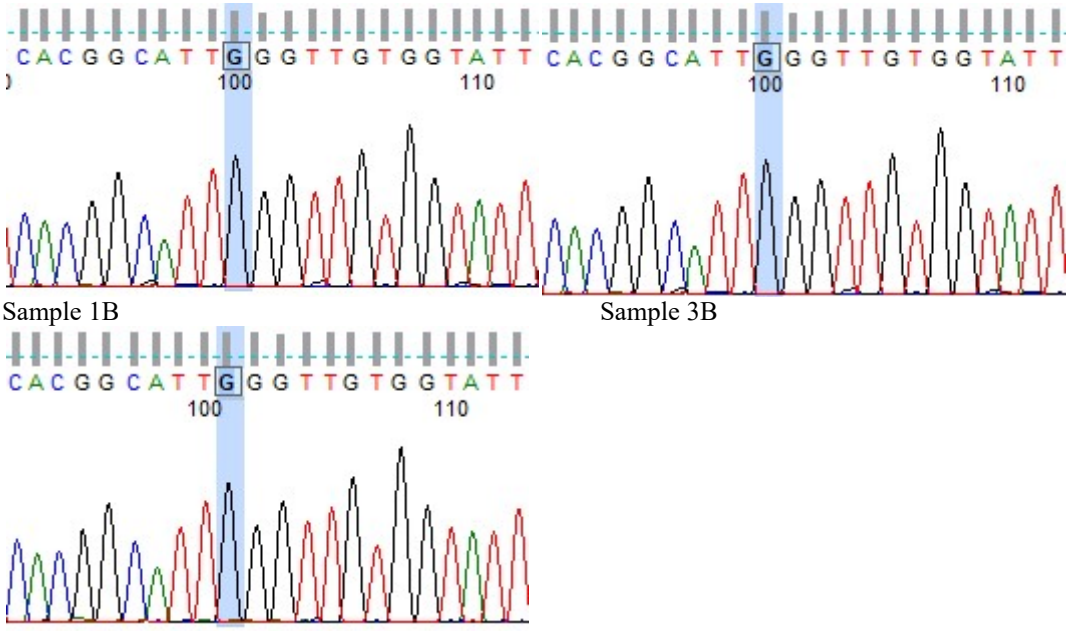


Figure (20) Chromatograms of Sanger DNA sequencing samples (1B,3B and 8B) of *GyrB* leucine 474 changed from A to G and wild type of *GyrB* gene not shown

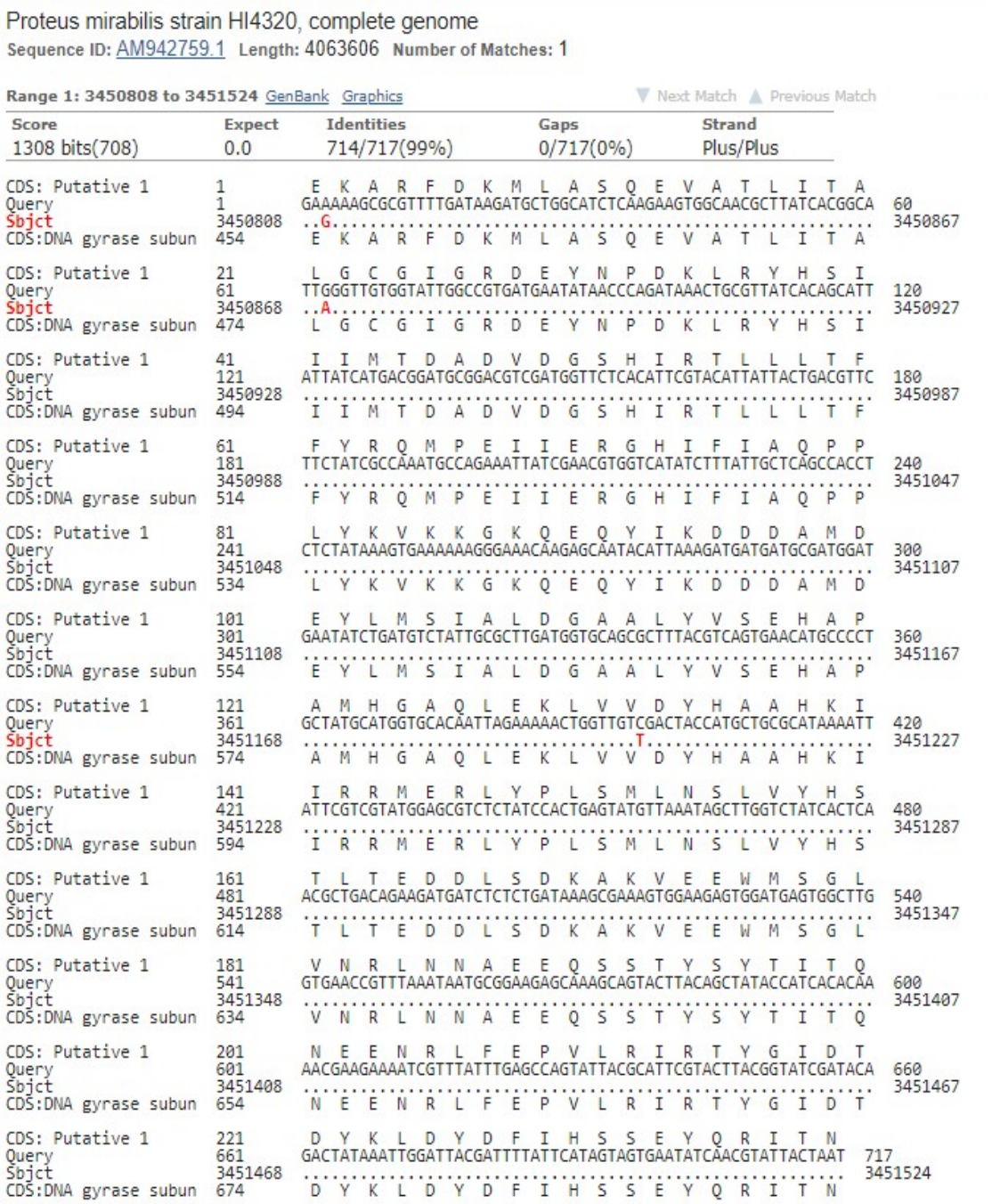


Figure (21) Alignment of *P. mirabilis GyrB* with reference *P. mirabilis* HI4320 strain

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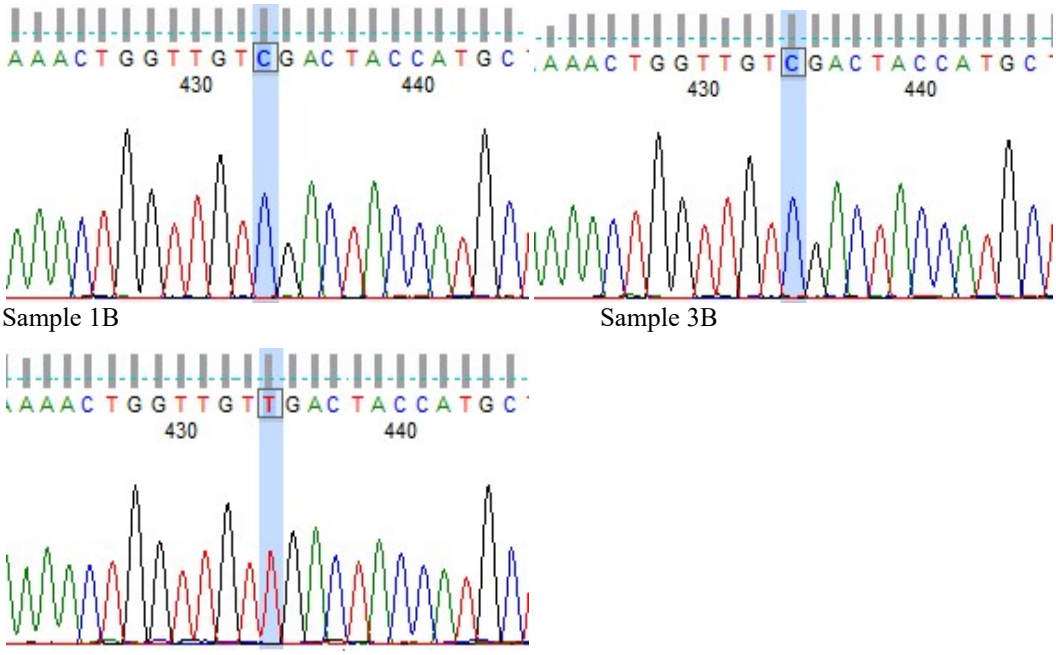


Figure (22) Chromatograms of Sanger DNA sequencing samples (1B and3B) of *GyrB* valine 585 changed from T to C and wild type of *GyrB* gene

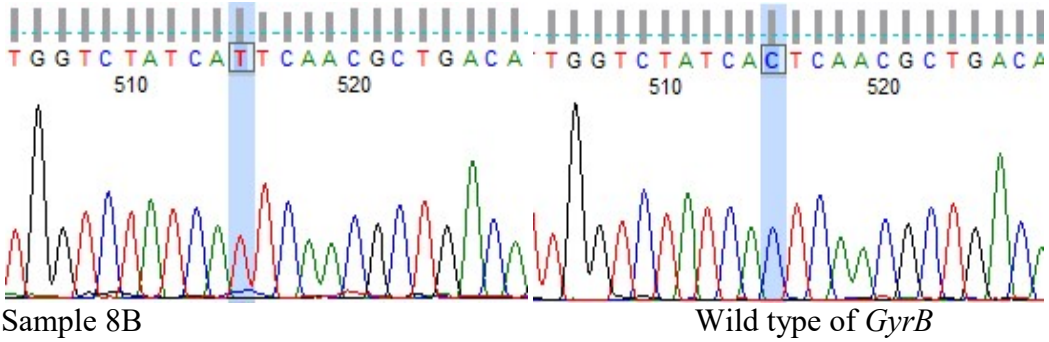


Figure (23) Chromatograms of Sanger DNA sequencing sample 8B of *GyrB* histidine 612 changed from C to T and wild type of *GyrB* gene

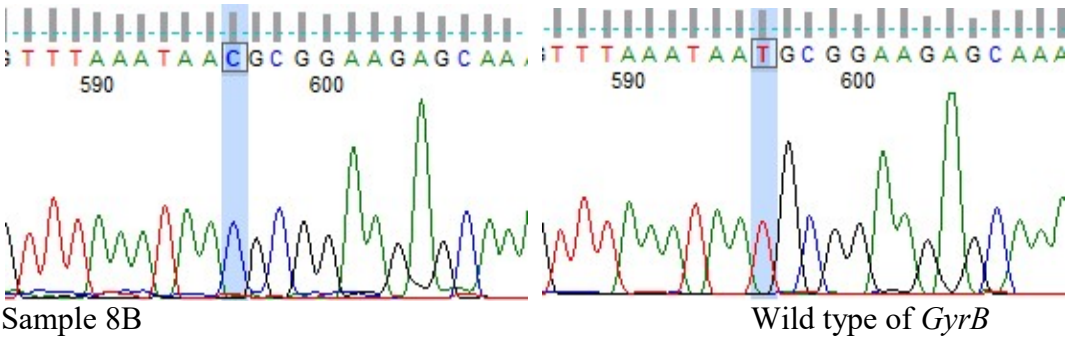


Figure (24) Chromatograms of Sanger DNA sequencing sample (8B) of *GyrB* asparagine 639 changed from G to Tand wild type of *GyrB* gene

Sequencing of *ParC* QRDR of *P. mirabilis* alignment with reference *P. mirabilis* HI4320 strain in GenBank database NCBI by nucleotide blast as shown in (figure 28) revealed mutations at codon 84 and silent mutation at codon 81 and 116 (Table 2). Serine 84 to isoleucine at resistance strains (1C and 3C) substitution (G 251 T) (figure 25 and 29), histidine 81 in sensitive strain (8C) substitution (C 243 T) (figure 26) and codon 116 proline in resistance strain (3C) substitution(A 348 T) (figure 27).

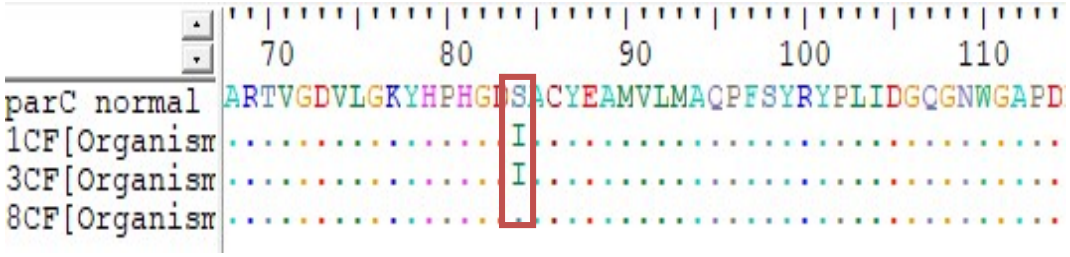
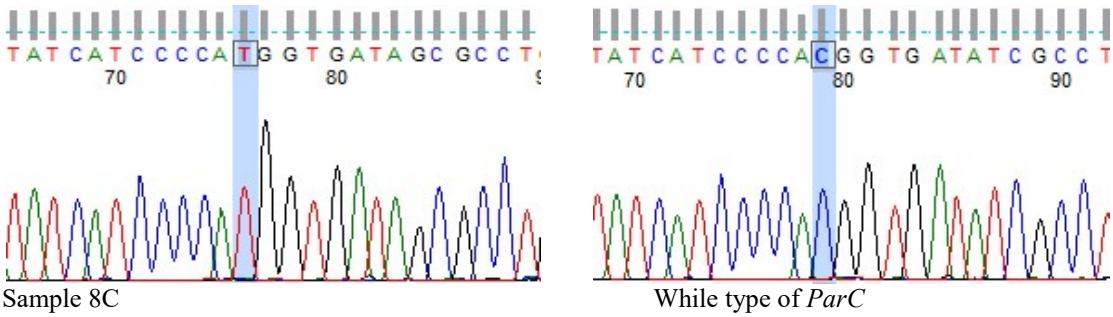
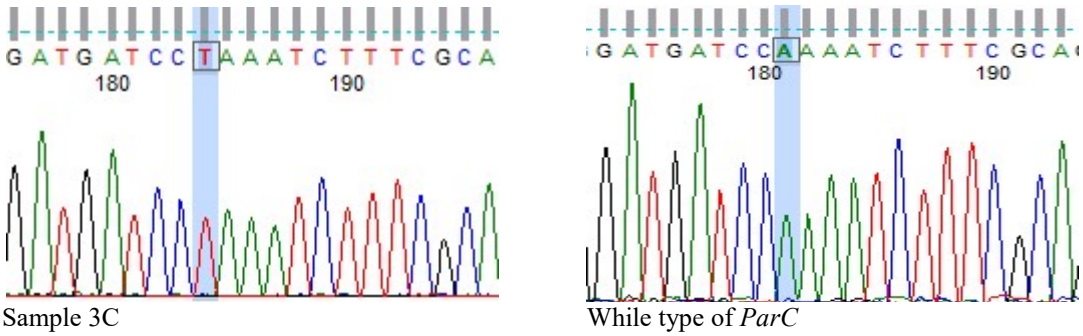


Figure (25) *ParC* amino acid changed codon 84 serine to isoleucine (AGT- ATT). Analyses was done by BioEdit alignment editor v7.2.5



Sample 8C While type of *ParC*

Figure (26) Chromatograms of Sanger DNA sequencing sample (8C) of *ParC* histidine 81 changed from C to T and wild type of *ParC* gene



Sample 3C While type of *ParC*

Figure (27) Chromatograms of Sanger DNA sequencing sample (3C) of *ParC* proline 116 changed from A to T and wild type of *ParC* gene

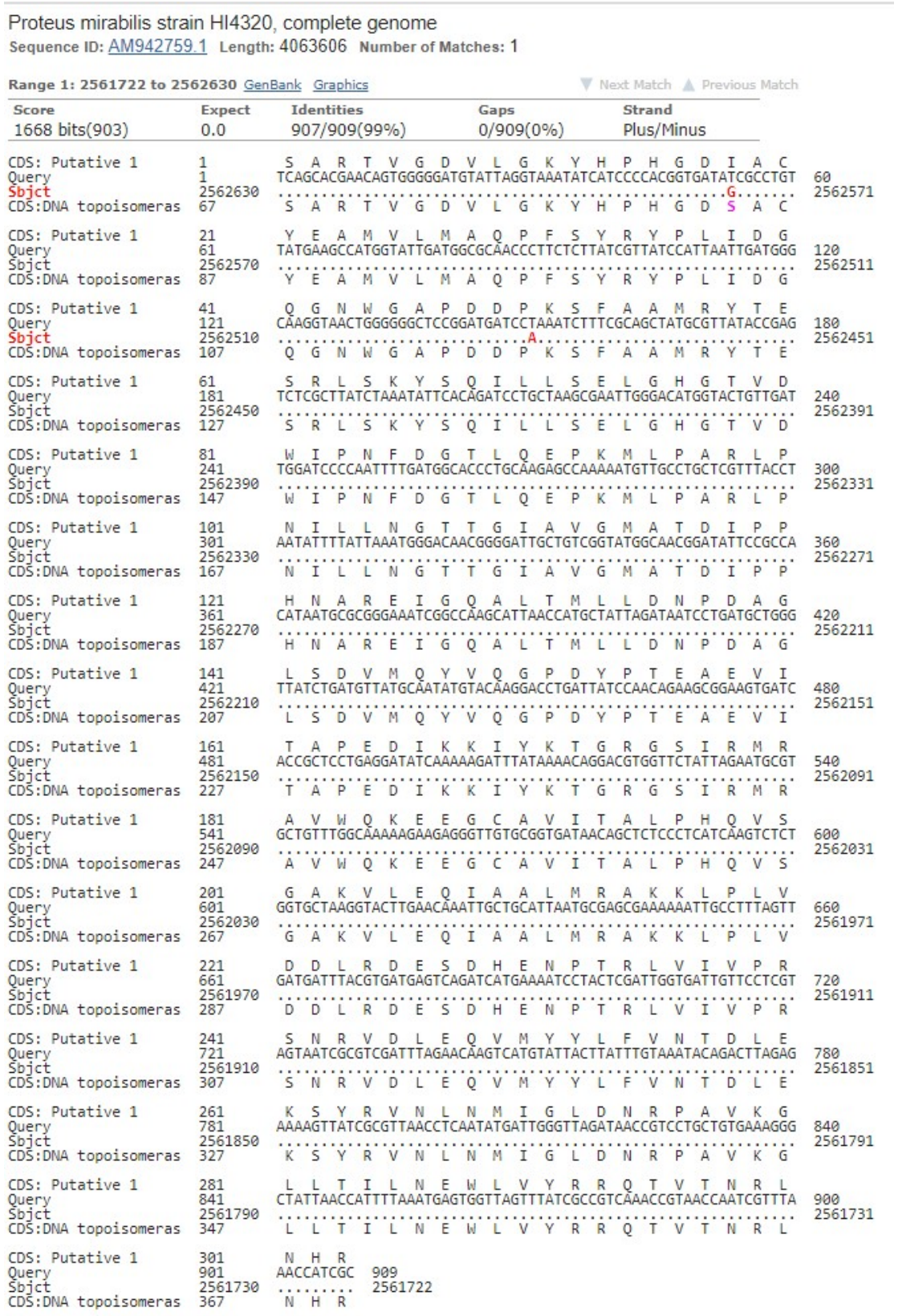
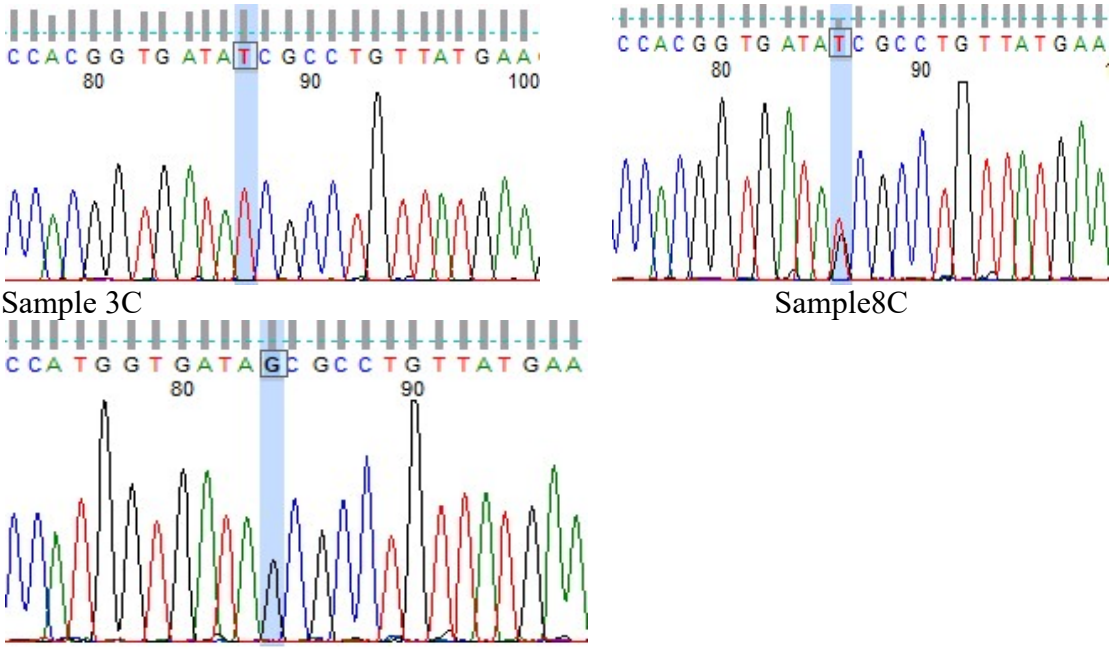


Figure (28) Alignment of *P. mirabilis ParC* with reference *P. mirabilis* HI4320 strain



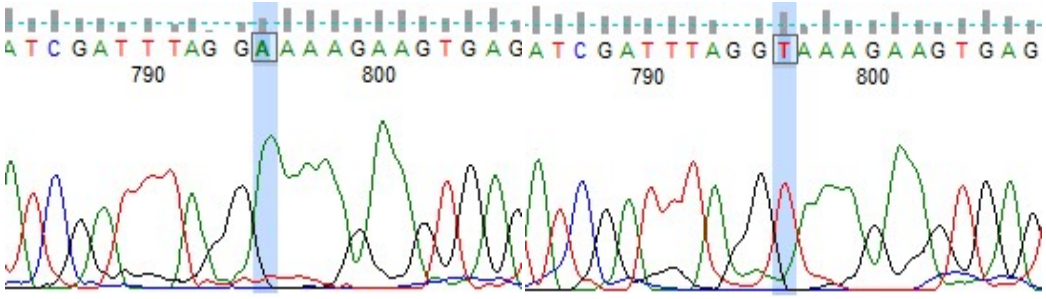
Sample 3C

Sample8C

While type of *ParC*

Figure (29) Chromatograms of Sanger DNA sequencing a. sample (3C and 8C) of *ParC* serine 84 changed from G to T isoleucine and wild type of *ParC* gene

Sequencing of *ParE* QRDR of *P. mirabilis* alignment with reference *P. mirabilis* HI4320 strain in GenBank database NCBI by nucleotide blast as shown in (figure 31) revealed mutations silent at following codons (469, 531 and 533) (Table 2). Codon (469) isoleucine in resistance stain (3E) and sensitive strain (8E) substitution (C 1407 T) (figure 32), codon (531) aspartic acid in resistance stain (3E) and sensitive strain (8E) substitution (C1593T) (figure 33) and codon (533) glycine in sensitive strain (8E) substitution (1 599 A) (figure 30).



Sample 8E

Wild type of *ParE*

Figure (30) Chromatograms of Sanger DNA sequencing sample (8E) of *ParE* glycine 533 changed from G to T and wild type of *ParE* gene

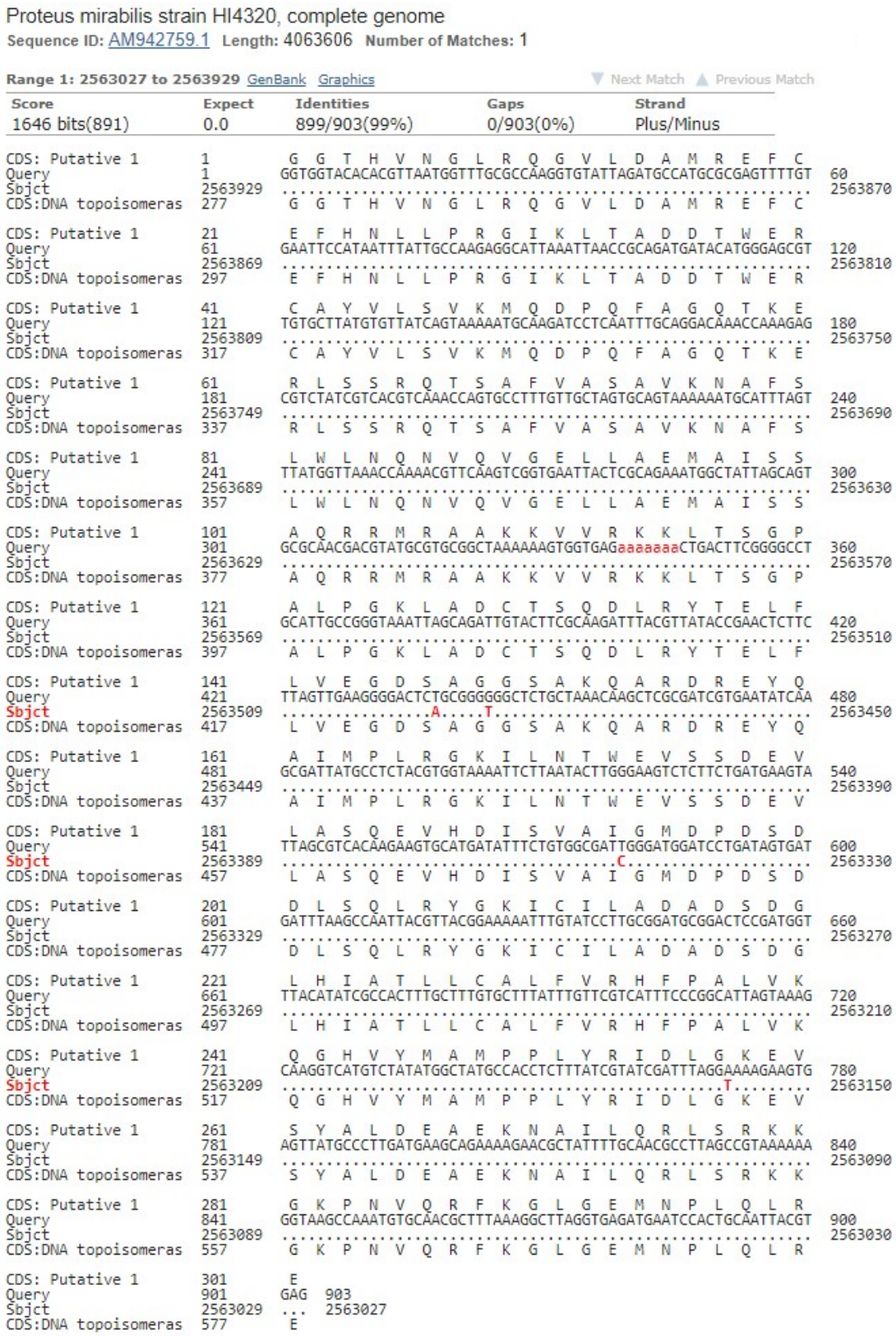
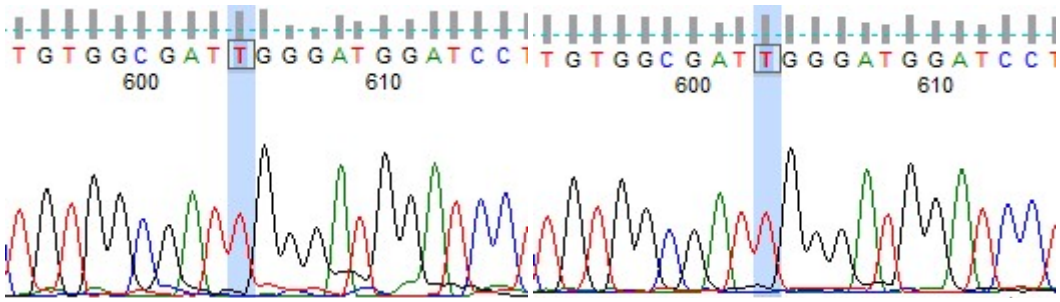
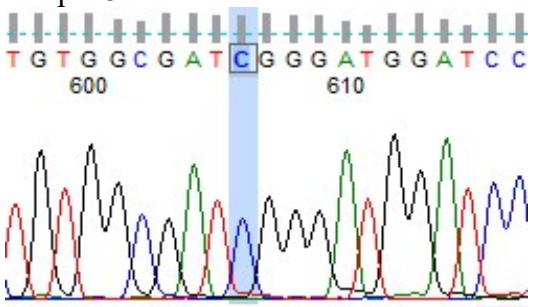


Figure (31) Alignment of *P. mirabilis ParE* with reference *P. mirabilis* HI4320 strain

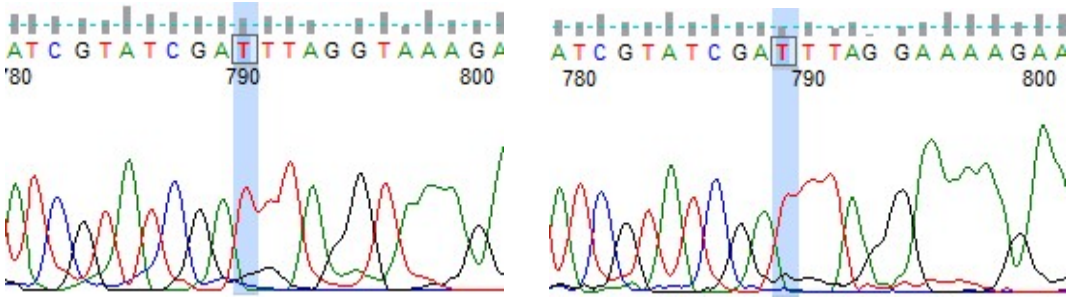


Sample 3 Sample 8

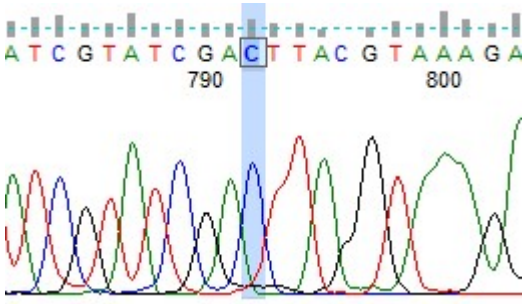


Wild type of *ParE*

Figure (32) Chromatograms of Sanger DNA sequencing sample (3E and 8E) of *ParE* isoleucine 469 changed from C to T and wild type of *ParE* gene



Sample 3E Sample 8E



Wild type of *ParE*

Figure (33) Chromatograms of Sanger DNA sequencing sample (3Eand 8E) of *ParE* aspartic acid 531changed from C to T and wild type of *ParE* gene

CHAPTER FIVE

DISCUSSION

The incidence of resistance to fluoroquinolones among aerobic Gram negative bacilli has increased during the last decade. Resistance to fluoroquinolones was first known as a problem in *Ps. aeruginosa* and other non-fermenting Gram-negative bacilli, but more recently has become well known among other *Enterobacteriaceae*. It has been suggested that this shifting is particularly related to the abused and excessive use of fluoroquinolones. For instant, reports of the emergence of fluoroquinolone resistant strains of *P. mirabilis* have not been fully recognized. Moreover, the genus *Proteus* are isolated from patients, especially from those with UTIs (Martínez *et al*., 1993; Pérez *et al*., 1993).

In this study the findings suggested high prevalence of UTIs among females comparing to males (53.5%: 46.5%) These findings were in agreement with previous study conducted in Sudan by Abd Elrahman *et al*. (2018) who recorded that female (50.5%): male (49.5%) and Murtada *et al*. (2014) female (55.5%): male (44.5%), in Iran by Arman *et al*. (2016) who record female (60%): male (40%). But did agreed with Otajevwo (2011) in Nigeria who noted that female (41.7 %): male (58.3%).

The study also confirmed that *P. mirabilis* was isolated from 120 (3.1%) patients with different ratio females (55):males (45) These findings were agreed with a previous study conducted by Hamdan *et al*. (2015) in Sudan who recorded that the prevalence of *P. mirabilis* among UTI patients was (66.6: 33.4). Similarly, Akinloye *et al*. (2006) mentioned that the infected males were (44.5) while the infected females were (54.6). Contrary to this observation, Omer (2015) in Sudan who recorded

that (males 55.6: females 44.4), Saleh and hatem (2013) in Saudi Arabia who record males(75): females (25),Otajevwo (2011) in Nigeria noted that the prevalence rate of the infection among males (66.6%): females (33.4%) was respectively. This is mostly due to the anatomy shortness of the female urethra distance that bacteria must travel to reach the bladder. Bacteria from fecal matter at the anal opening can be easily transferred to the opening of the urethra.

UTI commonly infected all ages, but the incidence increases with older age factors contributing to the higher incidence of UTI with age include urinary tract anomalies, changes in hormonal status, urinary incontinence, decline in the immune system, malnutrition, functional disability, and coexisting illnesses (Luiz *et al*., 2012). In this project UTI occurred highest in the age group (11 – 49) years this observation is supported by a variety of researchers globally; in Sudan Omer (2015) and Badri and Mohamed (2017), in Nigeria Oluremi *et al*. (2011) and Onoh *et al*. (2013).

UTI commonly is caused by a range of pathogens, but most commonly by *E. coli*, *K. pneumoniae*, *P. mirabilis*, *E. faecalis* and *S. saprophyticus (*Flores *et al*., 2015). Gram negative bacteria isolates were more prevalent (89.1%) than gram positive bacteria isolates (10.9%). Similar rate of isolation of gram negative and gram positive bacteria (85.9%:14.1%), was reported in Egypt by Mohamed *et al*. (2012) and (92.9 and 6.1%) in United Stat of America by Mcloughlin and Joseph (2003). This could be due to the presence of unique structure in gram negative bacteria which facilitate their attachment to the uroepithelial cells, multiplication, and tissue invasion.

The results obtained from this study suggested a frequency of (3.1%) of *P. mirabilis*, which is almost neighboring to the results obtained by Amir *et al*. (2017); (4%) and less than that announced by Abd Elrahman *et al*.

(2018); (6%). In the contrary in Sudan, Murtada *et al*. (2014) suggested low frequency of *P. mirabilis* among his target group (0.5%). Different studies worldwide appeared with different findings; in Nigeria (9.5%) by Onoh *et al*. (2013), in India (4.54%) by Sujatha and Nawan (2014), in China (3.39 %) by Yang *et al*. (2017) and in Italy De Francesco *et al.* (2007) also suggested the lowest frequency of *P. mirabilis* among all gram negative that capable for causing UTIs.

On the other hand, this results confirmed the existence of *E. coli* as the most dominant pathogens that caused UTIs among Sudanese patients (56.1%). This finding was totally agreed with the majority of reports that were received from Sudanese library (Omar, 2015, Murtada *etal*., 2014, Othman, 2007 and Elder, 2004). Moreover, this high percentage has been reported by many researchers globally; (47.5 %) in Egypt by Khalifa *et al*. (1987), (50.1 %) in Saudi Arabia by Ahmed and Ahmed (1995), (55.6 %) in Jordan by Farah and Murshidi (1996), (84 %) in Turkey by Arslan *et al*. (2005). The higher percentage of *E.coli* infection compared with the other organisms could be explained on the basis of their normal habitat in the intestinal tract that is why it is the most common organism founded.

In contrast, *K. pneumoniae* was isolated with a percentage following *E. coli* (18.0%). This finding is totally agreed with Badri and Mohamed, (2017) who announced for a percentage of (16.7) among Sudanese populations. Similar reports were received from Egypt by Mohamed *et al*. (2012), from Turkey by Yusel *et al*. (2006), from Pakistan by Gourshi (2005), from Ethiopia by Beyene and Tsegaye (2011), from Jordan by Almomani (2006). Rare studies from United Stat of America reported low frequency of *K. pneumoniae* among infected subjects (3.7%) (Mcloughlin and Joseph, 2003).

Ps. aeruginosa was isolated in (8.9%) of urine specimens, which was similar to the results obtained by Mohamed *et al. (*2012) from Egypt

(9.4). But low frequency of *Ps. aeruginosa* reported by Murtada *et al*. (2014) in Sudan among his target group (1%).

The results obtained from this study showed (2.2%) of *P. vulgaris*, which is almost nearby to the results obtained by Abd Elrahman *et al* (2018) (2%); and Murtada *et al*. (2014) (2.5%); among Sudanese populations. The result was different from Mcloughlin and Joseph (2003) in United Stat of America, suggested low frequency of *P. vulgaris* among his target group (1.2%).

Citrobacter spp. were isolated in (0.8 %) of urine specimens. This finding was agreed with Murtada *et al*., (2014) who reported (16.7%) among Sudanese populations. Similar reports were received from United Stat of America by Mcloughlin & Joseph (2003) who found *Citrobacter* spp. were (1.2%). In other hand high percentage recorded by Abd Elrahman *et al*. (2018) in Sudan who found *Citrobacter* spp. were (2%).

The study showed that *E. faecalis* was (9.9%) of isolated bacteria, similar reports were received from Sudan by Hamdan *et al*., (2015) who found the *Enteroccoci faecalis* was (12.8%). But this result was high rate compared with Sudanese researchers; (3.5%) by Murtada *et al*. (2014) and (4%) by Badri and Mohamed (2017). Also higher than Mcloughlin & Joseph (2003) from United Stat of America who found the *E. faecalis* was (3.7%).

The lower percentage of isolated bacteria in this study was *S. aureus* (0.4%). This finding was agreed with the common reports that were received; from Nigeria Kolawole *et al*. (2009) and Mcloughlin& Joseph (2003) from United Stat of America. Although it is variance with other studies received from; Sudan (13%) by Amir *et al*. (2017), from Egypt (7.8 %) by Mohamed *et al*. (2012) and from Nigeria (20.6%) by Onoh *et al*. (2013).

Ciprofloxacin is a recommended drug for the treatment of UTIs but a progressive increase in ciprofloxacin resistance has been seen in clinical isolates of the bacterium, the results confirmed the highly resistant of isolated bacteria to ciprofloxacin (71.7%) among Sudanese patients (56.1%). This finding was agreed with Badri and Mohamed (2017) from Sudan who found (79%) of bacteria were resistant to ciprofloxacin. In addition, low percentage of resistance has been reported by many researchers; (13.5%) in Sudan by Abd Elrahman *et al*. (2018) and (35.3%) in Nigeria by Onoh *et al*. (2013).

The results obtained from this study showed (30%) of *P. mirabilis* resistance to ciprofloxacin, this finding was agreed with Ana *et al.* (2000) from Brazil(30%), Rajivgandhi *et al*. (2018) from India (28%) and Kyung *et al*. (2011) from Korea (27%) of *P. mirabilis* resistance to ciprofloxacin. Different studies appeared with different findings; in Sudan Amir *et al*. (2017) who found that no resistance to ciprofloxacin by *P. mirabilis*, in Japan(16%) by Saito *et al*. (2007), in Poland (40%) by Joanna *et al*. (2013) and in Taiwan by Wang *et al.,* (2014) who found that (68.7) of *P. mirabilis* reactance to ciprofloxacin. Generally, the possible reasons behind the resistance to ciprofloxacin in Sudan may be this antibiotic have been in use for a long period and must have been abused and as a result the organisms must have developed mechanisms of changing their mode of action.

Finally, the second part of the thesis (sequencing part). Mutations in the genes for the subunits *GyrA* and *ParC* of the target enzymes DNA gyrase and topoisomerase IV are important mechanisms of resistance in quinolone-resistant bacteria. The target enzymes also consist of the subunits *GyrB* and *ParE*.

P. mirabilis always mutated in *GyrB* (Ser 464 to Tyr or Phe) by (Saito *et al*., 2006). This amino acid is not present in *P. mirabilis GyrB* sequence

of clinical isolates but revealed silent mutations in the following codons (474) leucine, (585) valine, (612) histidine and (639) asparagine. Also *ParE* gene always mutated in (Val 364 to Iso) in *P. mirabilis* by (Weigel *et al*.,2002), *E. coli* (Leu 445 to His) and the viridans group of *streptococci* (Pro 424 to Gln) seems to suggest that the region spanning positions (424 to 460) in *ParE* confers quinolone resistance (Mclver *et al*., 2004; Gonzalez 1998). Sequence analysis of *ParE* gene fragments from the clinical isolates revealed silent mutations in the following codons (469) isoleucine, (531) aspartic and (533) glycine. However, no mutations in the corresponding region of *parE* were detected in either quinolone resistant or sensitive *P. mirabilis* isolate. The *ParE* do not play an important role in fluoroquinolone resistance among *P. mirabilis* as suggested by (Weigel *et al*., 2002).

Ciprofloxacin resistant *P. mirabilis* possessed the mutations in *GyrA* (Ser 83 to Ile), This amino acid changes are the same as those reported for fluoroquinolone resistant by (Saito *et al*., 2006; Weigel *et al.,* 2002) whom found that *P. mirabilis* mutations in *GyrA* (Ser 83 to Arg or Ile) , other studies appeared different mutations in *GyrA* with other bacteria; (Ser 84 to Leu) of *S. aureus* by Franz *et al* (1998), (Ser 83 to Leu) of *E. coli* by Varughese *et al*. (2018), (Ser 83 to Phe) of *M. bovis* by Lysnyansky *et al* . (2009).

P. mirabilis sequencing of *ParC* showed mutated in (Ser 84 to Ile), this result was agreement with many researches which proofed that *Proteus mirabilis* always mutated in *ParC* (Ser 84 to Ile) for fluoroquinolone resistant by Saito *et al*., (2006) and Weigel *et al.,* (2002). Also *E. tarda* was mutant in (Ser 84 to Ile) of *ParC* which associated with fluoroquinolone resistance in by Kim *et al*. (2010).

It is accepted that changes in the structure of the antibiotic targets DNA gyrase and DNA topoisomerase IV are one of the most significant

mechanisms in conferring a resistance to fluoroquinolone in gram negative bacilli (Lee *et al*., 2005). In *E. coli*, more than two mutations in both *GyrA* and *ParC* genes are necessary to obtain resistance to ciprofloxacin (Saito *et al*., 2006, Weigel *et al.,* 2002, Fàbrega*et al*., 2009 and Lee *et al*., 2005). Nevertheless, the situation in *P. mirabilis* is rather different from that in *E. coli*, since we found in this study only one or double mutation could lead to ciprofloxacin resistant.

In *P. mirabilis*, topoisomerase IV is a target of quinolones and mutations at residues (Ser 80 and Glu 84) of *ParC* contribute to decreased fluoroquinolone susceptibility (Weigel *et al*.,2002). Although *ParC* mutations always along with mutations in *GyrA* are needed to acquire resistance to quinolones (Weigel *et al*.,2002), one of clinical isolate in this study had mutations in *ParC* without *GyrA*, suggesting that *ParC* might not only be a secondary target for quinolones but is really as important as *GyrA* to cause a decreased susceptibility to fluoroquinolones in *P. mirabilis*.

Additionally, In *Acinetobacter baumannii* the silent mutation in QRDR regions is enough for fluoroquinolone resistance (Ardebili *et al*., 2015). Nevertheless, the situation in *P. mirabilis* is rather different from that in *A. baumannii*, since we found in this study sensitive strain have silent mutation in *GyrB*, *ParC* and *ParE*.

Direct *Hinf*I digestion of PCR amplified have been used by many researchers to screen *Gyr*A and *ParC* genes mutations in different bacteria; *S. pneumoniae* at positions serine (83) of *GyrA* and serine (79) of *ParC* by (Stewart *et al*., 1999 and Rodrigo *et al*., 2004), *A. baumannii* at positions serine (83) in *GyrA* and (80) of *ParC* by (Shang *et al*., 2009; Ahmed and Sebastian 2004; Vakili *et al*., 2014) and *N. gonorrhoeae* at positions Ser (91) of *GyrA* by (David *et al*., 1998) significantly associated with ciprofloxacin resistance.

Direct *Hinf1* digestion of PCR amplified have been used to screen *GyrA* and *ParC* mutations in *P. mirabilis.* Mutation at codon (83) of the *GyrA* gene and (84) of *ParC* results in the loss of natural *Hinf*1 site was identified. The results indicated that all samples mutated at serine (83) of *GyrA* and serine (84) of *ParC.* When dealing with sequencing, two out of the three sequenced *GyrA* appeared as non-mutated at Ser (83) while one sample of the other three sequenced *ParC* resulted as free from any mutations at ser (84). These may have been attributed to the reason that serine (83) of *GyrA* and serine (84) of *ParC* in *Proteus mirabilis* consisted of (AGC) which is almost different from serine in other bacteria (TCC). Thus the loss of natural *Hinf*1 site (5'…GANTC…3') will be resulted.

CHAPTER SIX

Conclusion and Recommendations

6.1 Conclusion

The study concluded that:

6.1.1 Urinary tract infection is commonly caused by a range of pathogens, but most commonly by *E. coli* (56.1%) followed by *K. pneumonia* (18.0%), *E. faecalis* (9.9%), *Ps. aeruginosa* (8.9%), *P. mirabilis* (3.1%), *P. vulgaris* (2.2%), *Citrobacter* spp. (0.8%), *S. epidermidis* (0.6%) and *S. aureus* (0.4%).

6.1.2 High frequency of UTI among females compared to males (53.5%: 46.5%).

6.1.3 High frequency of urinary tract infection (53.6%) was detected in age group (11 – 49 years).

6.1.4 High resistant of all isolated bacteria to ciprofloxacin among Sudanese patients (71.7%), whilst *Proteus mirabilis* shown (30%) resistant to ciprofloxacin.

6.1.5 (33.3%) of ciprofloxacin resistant *Proteus mirabilis* showed mutations in *GyrA* (Ser 83 to Ile).

6.1.6 (66.6%) of ciprofloxacin resistant *Proteus mirabilis* showed mutations in *ParC* (Ser 81 to Ile).

6.1.7 Ciprofloxacin resistant and sensitive *Proteus mirabilis* revealed silent mutations in *GyrB* gene at codon 474 leucine, 585 valine, 612 histidine and 639 asparagine.

6.1.8 Ciprofloxacin resistant and sensitive *Proteus mirabilis* revealed silent mutations in *ParE* gene at codon 469 isoleucine, 531 aspartic and 533 glycine.

6.1.9 Only one or two mutations in both *GyrA* and *ParC* genes of *Proteus mirabilis* are necessary to obtain resistance to ciprofloxacin.

6.1.10 *Proteus mirabilis ParC* gene is important as *GyrA* gene to cause ciprofloxacin resistance.

6.1.11 In *Proteus mirabilis* the silent mutation in QRDR regions is not enough for ciprofloxacin resistance.

6.2. Recommendations

6.2.1 Further studies are needed to identify ciprofloxacin resistance genes DNA gyrase (*GyrA* and *GyrB*) and topoisomerase IV (*ParC* and *ParE*) and efflux bump in outer membrane proteins of *Proteus mirabilis*.

6.2.2 All isolated bacteria should be screened by disk disc diffusion test before treatment.

6.2.3 Establishment of antibiotic policies (ciprofloxacin) and treatment guideline.

6.2.4 Promoting careful use of ciprofloxacin by health professionals, increasing public awareness through public education campaigns and checking over the counter sale of antibiotic.

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123. **Zunino P., Sosa V., Allen A. G., Preston A., Schlapp G. and Maskell D.J. (2003),** *Proteus mirabilis* fimbriae (PMF) are important for both bladder and kidney colonization in mice, *Journal of Microbiology*, vol. 149: Pages 3231-3237.

Appendix I

questionnaire

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

Shendi University

College of Graduate Studies and Scientific Research

Detection and sequencing of Ciprofloxacin Resistance DNA GyrA and GyrB, ParC and ParE genes among Proteus mirabilis Isolated from Urine Specimens of Sudanese Patients

Questionnaire for Requirement of PhD Degree

Date ……..……

ID number ……..……..…

Place of sample collection ……..……...…

Sex……

Age ……..……

Marital status : 1- married2- single 3- widow 4- divorced

Have you ever suffered from UTI ? 1- Yes 2- No 3- I Do't Know

Have you used ciprofloxacin recently? 1- Yes 2- No 3- I Do't Know

Lab Diagnosis

Isolated bacteria ……...…

Ciprofloxacin susceptibility test:

Sensitive …….…….Intermediate…….….Resistant…….…..

Appendix II

Reagents and Stains

Gram Stain (Cheesebrough, 2007)

Most bacteria can be differentiated by their Gram reaction due to differences in the cell wall structure into Gram positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol and Gram negative which after being stained with crystal violet lose their color when treated with acetone or ethanol and stain red with Saffranin.

Requirements

Crystal violet Gram stain (HiMedia)

To make 1 liter:

Crystal violet……..... ... . …20 g

Ammonium oxalate…… .....……9 g

Ethanol or methanol, absolute…… .....… ....….95 g

Distilled water…… to 1 liter

Lugol's iodine (HiMedia)

To make 1 liter:

Potassium iodide…….…..…….20 g

Iodine…….……..10 g

Distilled water…….…….. To 10 liter

70% alcohol

Absolute alcohol…….....…..70 ml

Distilled water…….....…….30 ml

Saffranin (HiMedia)

Method of Preparation

• The dried smear was fixed by heat.

• The fixed smear was covered with crystal violet for 30-60 minutes.

• The stain was washed off with clean water.

• All water was tipped and the smear covered with lugol's iodine for 30-60 minutes.

• The stain was washed off with clean water.

• 70% alcohol was rapidly applied for 10-20 seconds for decolourization and then washed rapidly with clean water.

• The smear then covered with Saffranin stain for 2 minutes.

• The stain was washed off with clean water, back of slide was cleaned.

• After air-dry, smear was examined microscopically by using X 100 lens.

Results

Proteus mirabilis appear as Gram negative rods.

Preparation of Turbidity Standard

- 1% v/v solution of sulpharic acid was prepared by adding 1 ml of concentrated sulfuric acid to 99 ml of water. Mix well.
- 1.17% w/v solution of barium chloride was prepared by dissolving of 2.35g of dehydrated barium chloride (BaCl2.2H2O) in 200 ml of distilled water.
- To make the turbidity standard 0.5 ml of barium chloride solution was added to 99.4 ml of the sulpharic acid solution. Mix well.
- A small volume of the turbid solution was transferred to screw-caped bottle of the same types as used for preparing the test and control inoculate (Chemie, 2014).

Oxidase test reagent

Dimethyl-p-phenylene diamine hydrochloride …….1.0 g

Distilled water …….100.0 ml

The reagent should be made fresh daily. It should not be stored longer than one week in the refrigerator.

If the preparation becomes darkened, discard. Tetramethyl-p phelylenediamine dihydrochloride (1%) is even more sensitive but it is more expensive and difficult to obtain.

Culture media

Preparation of Media (Chemie, 2014)

Blood agar

Formula in grams per liter (PH 7.2)

Nutrient agar…….1000 ml

Defibrinated blood……..…….50 ml

Preparation:

Autoclave the nutrient agar at 12 10C for 15 minutes. Cool to 45-50°C and add 50 ml of sterile blood aseptically. Rotate to mix thoroughly avoiding accumulation of air bubbles and pour immediately into sterile tubes or plates, i.e., before solidification.

CLED Agar (Cysteine Lactose Electrolyte Deficient)

Formula in grams per liter (PH 7.4)

Lactose.....10,00

Gelatin Peptone 4,00

L-Cysteine 0,128

Bacteriological Agar 15,00

Casein Peptone 4,00

Beef Extract..... 3, 00

Bromothymol Blue 0,02

Preparation

Suspend 36 grams of the medium in one liter of distilled water. Soak 10-15 minutes and mix well. Heat slowly while stirring frequently boils for a minute. Sterilize in the autoclave at 121°C (15 lbs. of sp.) for 15 minutes. Pour into Petri dishes. When the medium is solidified, invert the plates to avoid excess moisture.

Nutrient agar
Formula in grams per liter

Peptone ……..1.0 g
Sodium chloride…….…... ..5 g
Beef extract ……..……...3 g
Agar ……20 g
Distilled water ……..…….1000 ml
pH ……6.8

Preparation:

Dissolve all components in distilled water except agar. Adjust the pH. Add the agar. Sterilize in autoclave at 121°C for 20 min.

Kligler Iron Agar

Formula in grams per liter

Peptone mixture 20,00

Sodium Chloride..... 5,00

Ferric Ammonium Citrate 0,50

Phenol Red 0,025

Lactose 10,00

Dextrose 1,00

Bacteriological Agar 15,00

Sodium Thiosulfate.....0,50

Preparation

Suspend 52 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation. Boil for one minute. Dispense into tubes and sterilize at 121º C (15lbs. pressure) for 15

minutes. Allow to cool in a slanted position so as to obtain butts of 1’5-2 cm. Depth. For greater accuracy, Kligler Iron Agar should be used on the day of preparation or melted and solidified before use.

Tryptophan Culture Broth

Formula in grams per liter (PH 7.5)

Casein Peptone 10,00

L-Tryptophan 1,00

Sodium chloride.....5,00

Preparation

Suspend 16,0 grams of medium in one liter of distilled water. Heat to boiling agitating frequently. Distribute in test tubes, 3 ml each. Close the tubes with cotton or with a plastic or metallic cap. Sterilize at 121º C (15 lbs. sp.) for15 minutes.

Simmons Citrate Agar

Formula in grams per liter (PH 7)

Ammonium Dihydrogen Phosphate 1,00

Dipotassium Phosphate..... 1,00

Sodium Chloride.....5,00

Sodium Citrate 2,00

Magnesium Sulfate.....0,20

Bacteriological Agar..... 15,00

Bromothymol Blue.....0,08

Preparation

Suspend 24,3 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation until completely dissolved. Dispense in tubes and sterilize in the autoclave at 121ºC (15 lbs sp.) for 15 minutes. Cool the tubes in a slanted

position so that the base is short (1-1,5 cm. deep). Alternatively, the media can be poured into petri plates.

Christensen's Urea Agar

Formula in grams per liter (PH 6.9)

Gelatin Peptone..... 1,00

Dextrose 1,00

Sodium Chloride.....5,00

Monopotassium Phosphate 2,00

Urea 20,00

Phenol Red.....0,012

Preparation

Dissolve 29 grams of the medium in 100 ml. of distilled water. Sterilize by filtration. Separately dissolve 15 grams of agar in 900 ml. of distilled water by boiling. Sterilize in autoclave at 121°C (15 lbs.sp) for 15 minutes. Cool to 50°C and add to the 100 ml. of the sterile Urea Agar Base. Mix well and dispense aseptically in sterile tubes. Leave the medium to set in a slanted position so as to obtain deep butts. At a pH of 6.8 to 7.0 the solidified medium should have a light pinkish yellow color. Do not remelt the slanted agar.

Mueller-Hinton Agar

Formula in grams per liter (PH 7.4)

Beef, infusion …….300.0g

Cas amino acids……17.5 g

Starch……..1.5g

Agar …….17.0g

Distilled water…….1000ml

Preparation

38.0 g of media was suspended in 100 ml distilled water. Sterilized by autoclaving at 15Ib pressure (121˚C) and poured in sterile petri dishes.

Molecular reagents

10 X TBE buffer

Formula in grams per liter

Tris base…….108 gm

Boric acid…….……..…..55gm

EDTA……..…...40 ml of 0.5M

Deionized water……..……..…1 liter

Preparation

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

1X TBE buffer

Formula in ml per liter

10 X TBE…….10 ml

Deionized water…….90 ml

Preparation

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

Ethidium bromide solution

Formula in grams per 1ml

Ethidium bromide……10 mg

Deionized water……I ml

Preparation

Twenty milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle.

Agarose gel

Preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

Appendix III

Primers

Oligo	***GyrA*_F**						
SEQ	5'- AGCGACATTGCCAGAGAAAT -3' (20 mer)						
GC%	**MW**		**Yield**		**Scale**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**	**(umoles)**		
45.00	6428.2	0.0	5.5	25.0	0.025	57.88	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***GyrA*_R**						
SEQ	5'- CACCGACTGCATCACGTTT -3' (19 mer)						
GC%	**MW**		**Yield**		**Scale**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**	**(umoles)**		
52.63	6047.0	0.0	5.3	25.0	0.025	58.77	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***GyrB*_F**						
SEQ	5'-GGCAAAACAAGGGCGTAA -3' (18 mer)′						
GC%	**MW**		**Yield**		**Scale**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**	**(umoles)**		
50.00	7138.6	0.0	6.6	23.0	0.025	56.21	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***GyrB*_R**						
SEQ	5'-GCCCCTTCTTCAATCAGGTT -3' (20mer)						
GC%	**MW**		**Yield**		**Scale**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**	**(umoles)**		
50.00	6227.0	0.0	5.3	23.0	0.025	57.78	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***ParC*_F**						
SEQ	5'-CAGCGTCGTATCGTCTATGC -3 '(20mer)						
GC%	**MW**		**Yield**		**Scale (umoles)**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**			
55.00	6206.0	0.0	4.9	21.0	0.025	58.68	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***ParC*_R**						
SEQ	5'-CGGCGTAATACTTTTTCTAAGC -3 (21mer)						
GC%	**MW**		**Yield**		**Scale (umoles)**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**			
40.91	6.206	0.0	4.5	21.0	0.025	55.92	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***ParE*_F**						
SEQ	5'-GGAAGGAGGCGATTTACTCA -3 '(20mer)						
GC%	**MW**		**Yield**		**Scale (umoles)**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**			
50.0	6115.2	0.0	5.1	25.0	0.025	57.02	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Oligo	***ParE*_R**						
SEQ	5'-GGATCAAGCGTTGTCTCACG -3 (20mer)						
GC%	**MW**		**Yield**		**Scale (umoles)**	**Tm (c)**	
	Calculate	**Measured**	**OD**	**nmol**			
55.0	6151.0	0.0	5.7	25.0	0.025	59.28	
vol.for 100pmol/ul		**Purification**			**Modification**		
250.0		Desalt					

Appendix IV

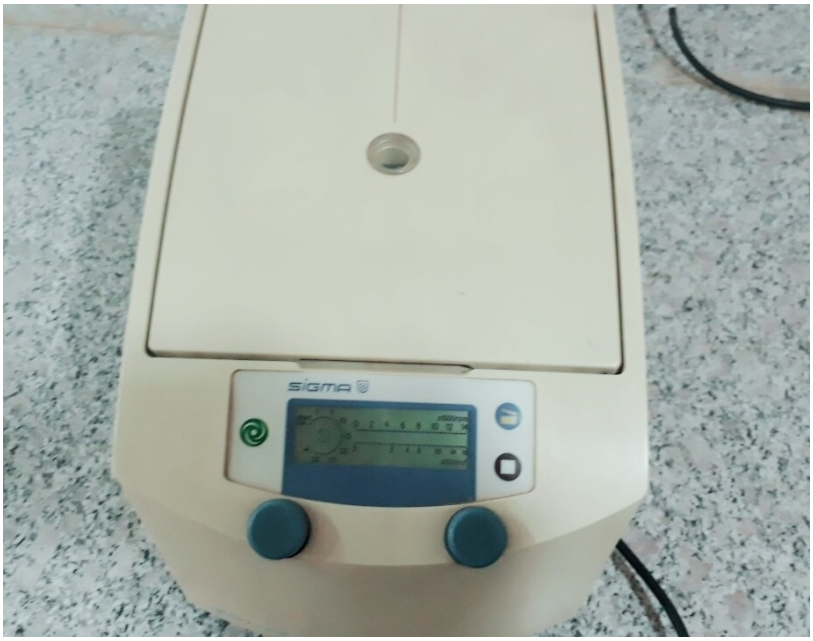


Figure (34) Sigma 1-14 Germany Microcentrifuge Device

Figure (35) CLASSIC K960 ChinaThermocycle Device

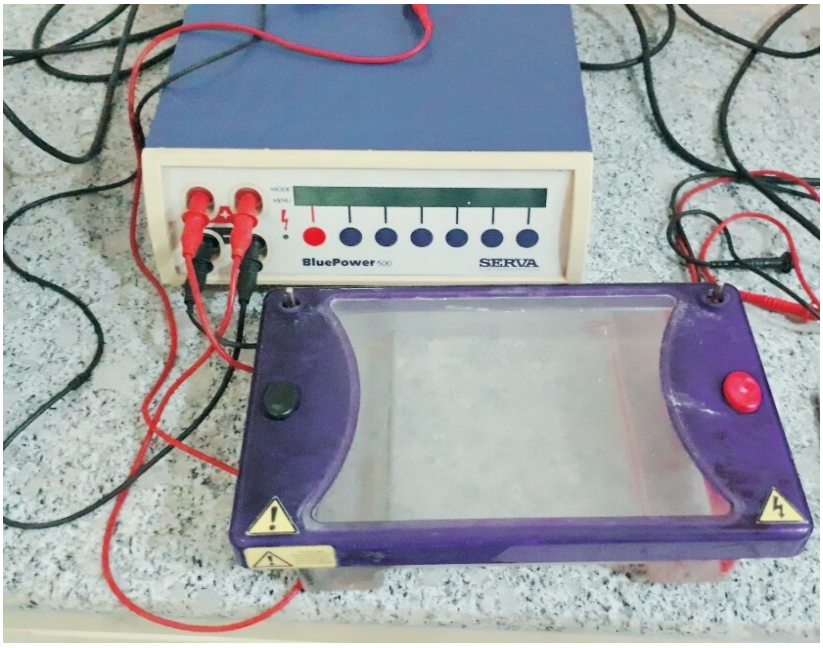


Figure (36) Gel Electrophoresis and Power Supply Device

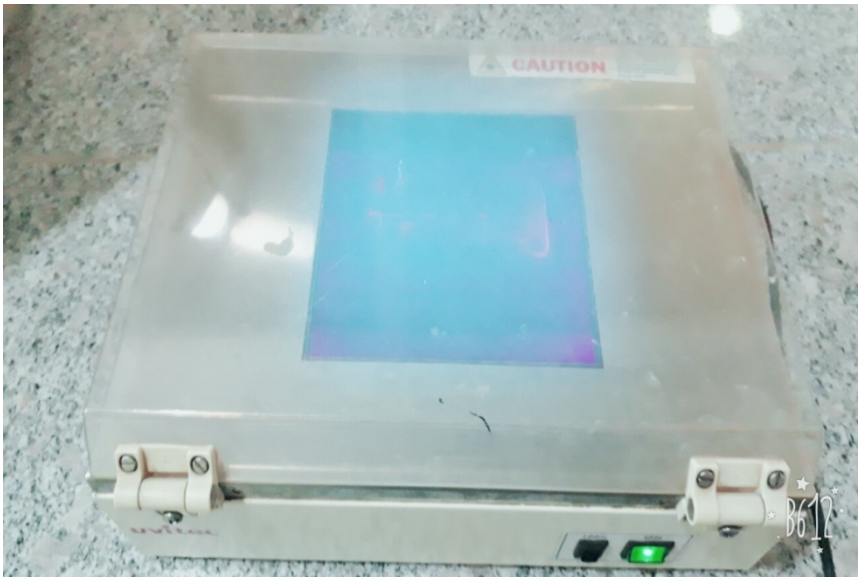


Figure (37) UV Light Transilluminater Device

Appendix V

Maxime PCR PreMix Series

Research Use Only

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20℃; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl . Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

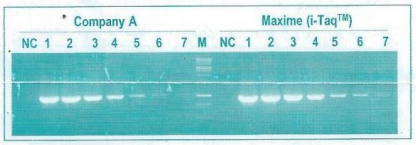


Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC**, Negative control

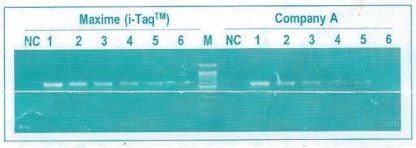


Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC**, Negative control

iNtRON BIOTECHNOLOGY
www.intronbio.com / info@intronbio.com
T. (0505)550-5600 / F. (0505)550-5660

Appendix VI

For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

SiZer™ DNA Markers

DESCRIPTION

iNtRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded RNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of double-stranded DNA from 60~10,000bp base pairs. The SiZer™ DNA Markers consist of 7 ~ 15linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EtBr) or other DNA stains.

CHARACTERISTICS

- Ideal for determining the size of DNA
- Stable for more than 12 months at - 20 ℃
- Ready to use without any handlings.

KIT CONTENTS

Product	Contents	Cat. No.
SiZer™-20 DNA Marker	0.3 ml	24071
SiZer™-50 plus DNA Marker	0.5 ml	24072
SiZer™-100 DNA Marker	0.5 ml	24073
SiZer™-1000 DNA Marker	0.5 ml	24074
SiZer™-1000 plus DNA Marker	0.5 ml	24075
SiZer™-15K DNA Marker	0.5 ml	24076
SiZer™- λDNA/HindIII DNA Marker	0.5 ml	24077

STORAGE

- Store at 4℃ and stable for more than 6 months. For more stable use, should be eliquoted and then stored at -20℃. (stable for more than 12 months)

GENERAL USE

- No DNase and RNase detected.
- Load 5 µl per each well of Agarose gel.

QUALITY CONTROL

Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.

The absence of nucleases is confirmed by a direct nuclease activity assay.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

NOTICE BEFORE USE

- Do not heat before loading
- For quantification, adjust the concentration of the sample to equalize, it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EtBr) or other DNA stains.

DETAIL INFORMATION

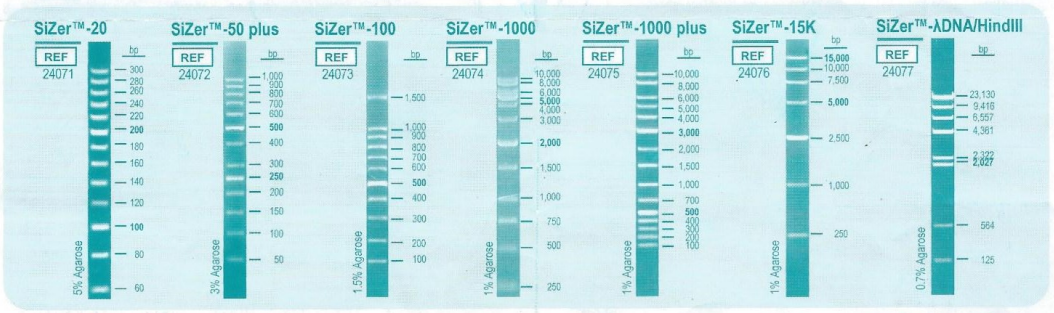
	Size range (bp)	Conc. (ng/µl)	Typical bands	Other bands	Loading Vol.	Band number	Contents
SiZer™ -20	60-300	128	100ng/5µl	40ng/5µl	5µl	13	60,80,**100**, 120,140, 160, 180,**200**,220, 240,260, 280,300
SiZer™ -50plus	50-500	128	100ng/5µl	40ng/5µl	5µl	13	50,100,150, 200, **250** 300, 400,**500**, 600, 700,800,900,1000
SiZer™ -100	100-1500	100	100ng/5µl	40ng/5µl	5µl	11	100,200,300, 400, **500**, 600, 700,800, 900, 1000,1500
SiZer™ -1000	250-10000	120	100ng/5µl	40ng/5µl	5µl	12	250, 500, 750, 1000,1500, **2000**, 3000,4000, **5000**, 6000,8000, 10000
SiZer™ -1000 plus	100-10000	144	100ng/5µl	40ng/5µl	5µl	15	100,200,300, 400,**500**, 700, 1000,1500,2000, **3000**,4000,5000, 6000,8000, 10000
SiZer™ -15K	250-15000	85	125ng/5µl	50ng/5µl	5µl	7	250, 1000, **2500**, 5000, 7500, 10000, 15000
SiZer™ - λDNA/HindIII	125-23130	100	350ng/5µl	-	5µl	8	125, 564, 2027, 2322, 4361, 6557, 9416, 23130

RELATED PRODUCTS

Product Name	Cat.No.
RedSafe™ Nucleic Acid Staining Solution (20,000x)	21411
DNA-spin™ Plasmid DNA Extraction Kit	17096/17097/17098
MEGAquick-spin™ Total Fragment DNA Purification Kit	17286 / 17287/17288
Maxime™ PCR PreMix (i-StarTaq)	25165
Maxime™ PCR PreMix (i-pfu)	25185

ELECTROPHORESIS

- The 5 µl of ladder DNA was loaded, and then electrophoresed for 1hr at appropriate concentration of gel



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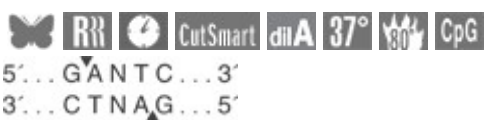
Appendix VII

HinfI

- 

Isoschizomers | Single Letter Code
Having supplied restriction enzymes to the research community for over 40 years, NEB has earned the reputation of being the leader in enzyme technologies. Working continuously to be worth of that distinction, NEB strives to develop enzyme of the highest purity and unparalleled quality.
  - Time-Saver™ qualified for digestion in 5-15 minutes
  - 100% activity in CutSmart® Buffer (over 210 enzymes are available in the same buffer) allowing for easier double digests.