1.1. **Introduction**

Escherichia coli are a genetically diverse species that includes many intestinal and extraintestinal pathotypes. Many of these have highly efficient and specialized mechanisms of colonization and pathogenicity, developed through the acquisition of virulence-associated genes and adaptation to their changing surroundings aided by mutations and natural selection. Many virulence-associated genes are localized on mobile genetic elements such as plasmids, bacteriophages and pathogenicity islands (PAIs). First described in uropathogenic *E. coli*, and now recognized in other pathotypes, notably enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), PAIs are distinct types of genetic element thought to have evolved from mobile genetic elements by horizontal gene transfer. Characterized by their large size (>10 kb), presence of virulence-associated genes and a G C content that is different from that of the rest of the genome, they are often flanked by repeat structures carrying fragments of other mobile and accessory genetic elements such as plasmids, bacteriophages and insertion sequence (IS). It is exchange of virulence genes between different bacteria that is largely responsible for the evolution of different bacterial pathotypes, and horizontal transfer plays a major role in the creation of new virulent clones (Johnson, 2002).

*E. coli*possess genes encoding many pathogenicity associated factors including adhesions, siderophores (i.e. aerobactin), capsule and toxins implicated in UTI pathogenesis. The published complete genome sequence of UPEC strain CFT073 reveals the presence of numerous pathogenicity-associated genes in UPEC, especially genes encoding potential fimbrial

adhesions, auto transporter iron sequestration systems as well as showing the acquisition of PAIs by horizontal gene transfer (Welch *et al*., 2002). Unlike *E. coli* O157:H7 and other EHEC strains, the UPEC CFT073 genome contained no genes for type III secretion system, phage or plasmid-encoded virulence genes. Individual pathogenicity-associated factors found in UPEC strains include adhesions, particularly fimbriae, which facilitate adherence and bacterial colonization. Many fimbriae, with different host receptor specificity, are expressed by UPEC strains. These include the mannose resistant P, M, S, F1C and fimbriae, which haemagglutinate erythrocytes in the presence of mannose. The type 1 fimbriae are common among Enterobacteriaceae, although in UPEC, the presence of type 1 fimbriae may increase their virulence for the urinary tract by promoting bacterial persistence and by enhancing the inflammatory response to infection (Wullt, 2003). The earliest described and most commonly associated adhesion in UPEC are Pimbriae, particularly the PapG adhesion. These fimbriae, encoded by the *pap* (pyelonephritisassociated pili) operon, recognize the disaccharide -D-galactosyl- (1–4)-D-galactose receptor, which is a very common P fimbriae group antigen. This enables it to bind to red cells, but also to uroepithelial cells in most of the population. There exists a strong relationship between the presence of P fimbriae and severity of infection, especially pyelonephritis. Also in UPEC are P-related sequences (Prs) which are closely related to P fimbriae but possess the F or PrsG adhesion which bind to galactosyl-*N*-acetyl (1–3)-galactosyl-*N*-acetyl(Wullt, 2003).

Toxins produced by UPEC include hemolysin and cytotoxic necrotizing factor 1 (CNF1) and secreted auto transporter toxin (Sat) which has been shown to have a cytopathic effect on various bladder and kidney cell lines

(Bahrani-Mougeot *et al.*, 2002).A high percentage of *E. coli* isolated from patients with pyelonephritis secreteshemolysin, which can be plasmid or chromosomally encoded. Additionally, on the chromosome of UPEC strains, the *hly* operon (*hlyCABD*) is often located near the P fimbrial genes on the same PAI. In strain 536, four PAIs have been characterized which carry many pathogenicity associated genes including two hemolysin gene clusters (PAI I536 and PAI II536), P-related fimbriae (PAI II536), S fimbriae (PAI II536) and the salmochelin (PAI III536) and yersiniabactin (PAI IV536) siderophore systems (Dobrindt *et al.*, 2002). The K15 capsule determinant of UPEC strain 536 is also found on a PAI (PAI V536) (Schneider *et al.*, 2004). Whilst the role of capsular antigens in UTI remains controversial, it is possible that they enable the bacteria to resist phagocytosis and survive in human serum or aid adherence of the bacteria to host cells.Interestingly, PAIs show considerable variability in their composition and structural organization. Some PAIs show genetic instability, having a tendency to be deleted from the chromosome, which can be influenced by environmental conditions, whereas others appear to be relatively stable (Middendorf*et al.*, 2004).

The severity of UTI produced by *E. coli* is expanded by the existence of a wide range of virulence factors. The most accepted theory today is that uropathogenic *E. coli* (UPEC) germinated from non-pathogenic strains by gaining new virulence factors via accessory DNA horizontal transfer often organized into clusters (pathogenicity islands) located at chromosomal locus (Bahalo*et al.*, 2013). The dominant of these virulence factors are sticky agents that help to colonization of bacteria in sites such as urethra or toxin that effect on the host. The UPEC possess adherence factors called pili or fimbriae, which allow them to successfully initiate infections.

Specific adhesion is mediated by bacterial proteins termed adhesions which may or may not be associated with fimbriae. *Pap* (pyelonephritis associated pili), *sfa* (S fimbrial adhesion) operons are most commonly found encoding P, S fimbriae respectively. (Ribeiro*et al.*, 2008). Besides bacterial adherence, several virulence factors may contribute to the pathogenicity of UPEC, including the production of hemolysin and aerobactin. (Arisoy *et al.*, 2005).

1.2. Rationale

E. coli generally exist as harmless commensal organisms on the mucous membranes of humans and other warm-blooded animals (Mora*et al.*, 2009). Intestine and extra intestinal variants are responsible for various diseases in these hosts (Herzer *et al.*, 1990; Whittam*et al.*, 1983). Extra-intestinal pathogenic *E. coli* (ExPEC) infections are now the most common cause of healthcare-associated infections (Rogers *et al.*, 2011: Kalantar *et al.*, 2008). These infections are caused by strains harboring numerous virulence factors located on plasmids or chromosomes (Muhldorfer and Hacker, 1994).Urinary tract infection (UTI), considered among the most common bacterial diseases that affect a large part of the world population (Kadri *et al.*, 2004). It is one of the most commonly occurring medical problems, causing considerable morbidity and healthcare costs. *E. coli* have become one of the most important causes of nosocomial and community-acquired infections especially urinary tract infection (UTI) and diarrhea (Orenstein and Wong, 1999).

The causative *E. coli* strain often can be found in the woman's fecal flora at the time of a UTI episode. This observation has suggested the 'fecal–perineal–urethral' hypothesis for UTI pathogenesis in women, according to which the host's own fecal flora is the immediate external reservoir from which *E. coli* strains emerge to cause UTI.This phenomenon, and the finding that the most common O antigens among *E. coli*UTI isolates are also the most prevalent O antigens among fecal*E. coli* from healthy individuals, has suggested the 'prevalence' hypothesis, which posits that UTI occurs when ordinary fecal*E. coli* are in the right place at the right time in sufficient numbers to enter the urinary tract and cause infection. In

contrast, the statistically greater prevalence among UTI-source *E. coli*, compared with fecal*E. coli*from healthy hosts, of phylogenetic group B2, certain O antigens, and suspected or proven virulence factors such as adhesions, siderophores, toxins, and polysaccharide coatings, has suggested the ‘special pathogenicity ’ hypothesis(Moreno*et al*.,2006).

Up to my knowledge there is no previous published data about detection of UPEC virulence genes in Sudan so for the first time in Sudan we went to compare the UPEC virulence genes isolated from patients suffering from UTI and diarrhea.

1.3. Objectives

1.3.1. General Objective

To genotypic detection of thevirulence factors ofuropathogenic*E. coli*isolate from diarrheic and urinary tract infected patientsin Khartoum State by using multiplex PCR.

1.3.2. Specific objectives

1- To isolate *E. coli* from diarrheic and urinary tract infection patients.

2- To screen the isolated *E.coli* for the antimicrobial susceptibility testing to most commonly used antibiotics against UTIs and diarrhea.

3- To characterize the virulence genes of UPEC (*fim, pap, sfa, aer and hly*)isolated from urine and diarrhea samples by multiplex PCR.

4- To compare between thepresences of UPEC virulence genes in urine and diarrhea samples.

5- To compare the presence UPECvirulence genes with gender, age group and antimicrobial susceptibility testing.

2. Literature Review

2.1. Escherichia coli

Since its first description by Dr Theodore Escherich in 1885, *E. coli* is the most extensively studied bacterial species. Much of the knowledge of bacteria at a molecular and cellular level has been obtained through studies with *E. coli*, particularly using the strainK12, isolated in 1922 from a patient with diphtheria. The complete genome sequence of a strain of *E. coli* K12 has been published, revealing the degree of genome plasticity, as indicated by the presence of phage remnants and insertion elements (Blattner *et al*., 1997).

The genus *Escherichia* includes motile and non-motile bacteria which belong to the family Enterobacteriaceae (Edwards and Ewing, 1972). They are Gram-negative, oxidase negative, non-spore-forming, rod-shaped bacteria, facultative anaerobes, which are often motile by peritrichously arranged flagella. They are capable of fermenting a wide variety of carbohydrates with production of both acid and gas, although anaerogenic biotypes exist. Rapid fermentation of lactose is a characteristic feature of many strains. Other genera within the family Enterobacteriaceae (*Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*), which share this ability to ferment lactose rapidly (typically within 24 h), are collectively termed coliform bacteria. Besides *E. coli*, other species belonging to the genus *Escherichia* are *Escherichia blattae*, *Escherichia fergusonii*, *Escherichia hermannii* and *Escherichia vulneris*. The species *Escherichia adecarboxylata* has since been assigned to the genus *Lecercia*, and a new species, *Escherichia albertii*, has been described (Abbott *et al*., 2003). With the exception of *E. blattae*, which has been isolated from the

cockroach intestine, but not from human clinical specimens, the primaryhabitat of *E. coli* and the other species is the gastrointestinal tract of humans and other warm-blooded animals where they generally exist as harmless commensal organisms. They can also occur in water, food and soil, but this is invariably the result of fecal contamination. Although most *E. coli* strains are harmless, there are others that cause disease in humans and animals that have evolved to become important pathogens in their own right. Clinically, two distinct types of pathogenic *E. coli* are recognized. One group commonly called extraintestinal pathogenic *E. coli* (ExPEC) includes those *E. coli* associated with newborn meningitis (NBM) or sepsis and urinary tract infections (UTIs).The second group termed intestinal pathogenic *E. coli* (IPEC) includes *E. coli* responsible for a range of distinct classes of diarrhoeal disease.

Multilocus enzyme electrophoresis (MLEE) and sequencing of the *malate dehydrogenase* gene (*mdh*) indicate that pathogenic strains of *E. coli* have arisen many times and that they do not have a single evolutionary origin within the species (Pupo *et al.*, 1997). Many prophage elements present in the *E. coli* O157 Sakai genome indicate that bacteriophages have played an important role in the emergence of this pathogen and, possibly, other pathotypes of *E. coli* (Ohnishi *et al.*, 2001). Although thought to be uncommon, genome rearrangements within species have been reported to be very frequent in human clinical isolates (Hughes, 2000). Comparison of genome size within a species also enables the degree of divergence between strains to be assessed. In strain MG1655 of *E. coli* K12 the chromosome is 4.6Mb (Blattner *et al.*, 1997), whereas genome sizes of 5.4Mb and 5.5Mb have been reported for *E. coli* O157:H7 strain EDL933 (Perna *et al.*, 2001) and the O157:H7 Sakai strain (Hayashi *et al.*, 2001),

respectively. The complete genome sequence of *E. coli* K12 strain MG1655 has provided a benchmark against which other *E. coli* strains have been compared. Comparison of *E. coli* O157:H7 EDL933 against this K12 strain has highlighted that lateral gene transfer in this O157:H7 strain has been more extensive than was initially expected (Perna *et al*., 2001). DNA sequences found in MG1655 but not EDL933 were designated K islands (KI) and DNA sequences in EDL933 but not MG1655, O islands (OI). There are 177 OI and 234 KI greater than 50 bp in length. Whilst they both share a common 'backbone' sequence of 4.1Mb (Perna *et al*., 2001), since the divergence of O157:H7 from K12 about 4.5 million years ago (Reid *et al*., 2000), this O157:H7 strain has acquired 1387 new genes (Perna *et al*., 2001). Many of these are putative virulence factors, while others are genes encoding alternative metabolic capacities as well as several prophages (Perna *et al*., 2002). Whilst the amount of apparent horizontal transfer in MG1655 is not as high as that observed in EDL933, there is evidence that the genome of MG1655 also contains DNA obtained through horizontal transfer (Blattner*et al*., 1997).

2.2. Pathologyof*E. coli*andclinicalfeaturesofInfections

Table (I) Different virotypes of *E.coli* causing human infection (Eduardo, 2001)

Type of *E. coli*	Disease	Virulence factors
Enterotoxigenic (ETEC)	Watery to cholera-like diarrhea	Heat-labile toxin (LT), heat-stable toxin (ST), colonization factors (CFs)
Enteroinvasive (EIEC)	Watery diarrhea to dysentery	Ipas, type III secretion (Mxi and

		Spa), VirG/IcsA
Enteropathogenic (EPEC)	Watery diarrhea	Esps, type III secretion (Sep and Esc), intimin, Tir, and BFP
Enterohemorrhagic (EHEC)	Hemorrhagic colitis, hemolytic uremic syndrome (HUS)	Above EPEC factors and Shiga toxin, hemolysin
Enteroaggregative (EAEC)	Watery to mucoid diarrhea	AAF adhesions, EAST-1, Pet, Pic, hemolysin
Diffusely adhering (DAEC)	Watery diarrhea	F1845 and AIDA-I fimbriae
Uropathogenic (UPEC)	Urinary tract infections	Type I pili, P pili, Afimbrial adhesions (Afa), hemolysin, CNF-1
Septic (SEC)	Neonatal sepsis, meningitis	Capsule, type I pili, S-fimbrial adhesion, IbeA and IbeB (invasion proteins)

2.2.1. Extraintestinal Pathogenic *E. coli*

Unlike *E. coli* responsible for diarrhoeal disease and life-threatening infections, ExPEC can either behave as harmless inhabitants of the human intestine or become serious pathogens when they enter the blood, cerebrospinal fluid (CSF) or urinary tract. Certain *E. coli* strains are responsible for classic syndromes such as UTIs, bacteremia and neonatal meningitis. This has given rise to particular strains, often characterized by specific O:K:H serotypes being classified as uropathogenic *E. coli* (UPEC)

or meningitis-associated *E. coli* (MAEC). Some strains differ from commensal *E. coli* by the presence of pathogenicity-associated genes and particular clones of *E. coli* such as strains of O4:H5 which appear to show a greater propensity to cause UTI. The high affinity iron-uptake system mediated by the hydroxamate siderophore aerobactin, which can be chromosomally or plasmid encoded, is reported to be particularly prevalent among ExPEC, including those that cause septicemia, pyelonephritis and meningitis (Carbonetti *et al.*, 1986). However, *E. coli* is capable of infecting many anatomical sites and some strains demonstrate pathogenic versatility and the ability to cause infections other than the classic syndromes (Johnson and Russo, 2002).

2.2.2. Urinary Tract Infections

E. coli is a normal inhabitant of the gut micro flora. However, certain *E. coli* strains cause severe human Infections(Duriez *et al.*, 2001; Hilbert *et al.*, 2008). *E. coli* are common facultative anaerobic bacteria found in the gastrointestinal tract of warm-blooded animals and humans. Although most *E. coli* are harmless symbioses, pathogenic strains are associated with a range of diseases of zoonotic importance. Worldwide, about 150 million people are diagnosed with UTI each year, costing the global economy in excess of 6 billion US dollars (Akram *et al.*, 2007). Usually UTI is defined as the presence of multiplying microorganisms in the tract through which urine flows from the kidneys via the bladder to outside (Hackett, 2005). More precisely UTI is a condition where one or more structures in the urinary tract become infected after bacteria overcome its strong natural defenses. From a microbiological perspective, UTI can

happen anywhere along the urinary tract which includes bladder, kidneys, ureters and urethra (Simon, 2006).

Urinary tract infection is one of the most prevalent of human infections that establishes by *E. coli*. At least 20% of women experience an acute symptomatic urinary tract infection during their lives. The severity of the infection depends on virulence of infecting bacteria and susceptibility of their hosts. Urinary infections often occur in patients with anatomically and functionally normal urinary tract, and involve spontaneous ascent of bacteria from the urethra to bladder and in a few patients to kidney. Adhesion of *E. coli* to uroepithelium may protect the bacteria from urinary lavage, increasing their ability to reproduce and invade renal tissue. (Santo *et al.*,2006).

E. coli which generally originate from faces or the periurethral flora are responsible for most UTIs. After colonizing the periurethral area, these organisms may ascend the urinary tract from the urethra meatus or from the insertion of catheters and infect the bladder. In some cases, these organisms continue to progress through the ureters and cause acute pyelonephritis, involving one or both kidneys. Patients with acute pyelonephritis present with a range of symptoms including fever, flank pain and bacteriuria with or without diaphoresis, rigors, groin or abdominal pain and nausea and vomiting. Abdominal tenderness of one or both kidneys may be elicited on examination. In a small proportion of cases, including those with pyelonephritis, the bacteria may spread beyond the urinary tract and enter the blood. The group of *E. coli*, often called UPEC, which are responsible for acute and chronic UTIs are distinct from

the commensal *E. coli* found in the colon of humans and are represented by a few serogroups (O1, O2, O4, O6, O7 and O75).

2.2.3. Bacteremia and Meningitis-Associated *E. coli*

Although *E. coli* are able to colonize and infect the gastrointestinal and urinary tracts of humans, septicemia remains a relatively rare complication of *E. coli* infections. Nevertheless, *E. coli* is one of the most common Gram-negative bacteria responsible for bacteremia in humans. Isolates of *E. coli* that infect the bloodstream often possess virulence factors that enable the organisms to circumvent the normal clearance mechanisms and evade the host immune response. These include a range of adhesions (P, S and M), the siderophore aerobactin and hemolysin which are found in other ExPEC (Agace *et al.*, 1993). The lipopolysaccharide (LPS) of *E. coli*, as with other Gram-negative bacteria, is an important pathogenicity factor which may cause fatal septic shock and disseminated intravascular coagulation. In response to the presence of *E. coli*, epithelial cells and cells of the host immune system have been shown to secrete many host-cell factors including interleukins (IL), tumor necrosis factor (TNF) and activators of the complement cascade. Epithelial cells are capable of producing a variety of cytokines in response to bacterial stimuli. Epithelial cells originating from the human urinary tract have been shown to produce IL-6, IL-1 and IL-8, whereas human peripheral blood monocytes additionally produce IL-1and TNF-in response to the presence of *E. coli*. Whilst *E. coli* bacteremia can occur in UTI, especially when the tract is obstructed, studies suggest a relationship between the magnitude of *E. coli* bacteremia and the development of meningitis. (Agce*et al.*, 1993)

The most common Gram-negative organism responsible for meningitis during the neonatal period is *E. coli*. Sepsis and NBM are often associated with *E. coli* belonging to a limited number of serotypes, particularly those expressing the K1 capsular antigen (Korhonen *et al.*, 1985) e.g. O83:K1 and O7:K1 and especially O18:K1. Whilst few specific pathogenic determinants have been described for *E. coli* causing NBM, isolates have been shown to possess many factors, some of which are also found in UPEC strains and others that appear to be specific to this group. The polysialic acid homopolymer K1capsule is believed to increase serum survival by blocking complement activation and expression of this, together with production of aerobactin, is believed to be important for blood stream dissemination. Adherence is a critical step in the pathogenesis of *E. coli* meningitis. Factors involved in the binding of *E. coli* to brain microvascular endothelial cells (BMECs) include S fimbriae which are also important in UPEC pathogenesis (Pluschke *et al.*, 1983). Subsequent invasion is facilitated by various microbial determinants including invasion of brain endothelium (Ibe) proteins, which may promote the crossing of the blood–brain barrier. A primary determinant of this event is a high density of bacteremia, but how circulating *E. coli* cross the blood–brain barrier is not fully understood: one mechanism might involve transcytosis through the endothelial cells aided by specific pathogen–host-cell interactions. Another factor which may contribute or enhance invasion of BMEC by *E. coli* is outer membrane protein A (OmpA), which shows structural similarities to *Neisseria* outer membrane protein (Opa) and surface protein PIII (Prasadarao *et al.*, 1996). Another candidate protein necessary for the invasion of BMEC is a novel ibe10 protein found in CSF isolates of *E. coli*, which has been shown to interact

with endothelial cells, thus enhancing invasion by *E. coli* cells (Prasadarao *et al*., 1999). Whilst possession of P fimbriae is important in *E. coli* causing pyelonephritis, it is not thought to be relevant in strains responsible for NBM. Furthermore, whereas hemolysin and CNF-1 are less common in NBM strains, *ibe*10 and *sfa* genes are reported to be more commonly associated with meningitis strains compared with blood or commensal *E. coli* (Prasadarao *et al*., 1996).

2.2.4. Intestinal Pathogenic *E. coli*

Six distinct groups have been defined within IPEC commonly associated with intestinal disease: EIEC, enterotoxigenic *E. coli* (ETEC), EPEC, EHEC, enteroaggregative *E. coli* (EAggEC) and the diffusely adherent *E. coli* (DAEC). These diarrhoeagenic *E. coli* are described in more detail, along with aspects of the associated pathogenesis and their epidemiology (Donnenberg, 2002; Kaper *et al*., 2004).

Some of the virulence-associated genes and key features of the different pathotypes are presented in Table (2) In the past, pathogenic *E. coli* were defined by their serotype on the basis of somatic O and flagella H antigens and to a lesser extent the K antigens. Although this practice continues, it has become more common to define individual pathotypes based on their pathogenetic characteristics. This is particularly important because of the existence of strains with the same serotype that belong to different pathotypes based on their pathology and complement of specific virulence determinants, which is apparent with some strains of EPEC and EHEC.

Table (2) Summary ofpathogenicity-related characteristics of intestinal pathotypes of *E. coli*(Stephen and peter, 2006)

Pathotypes	Pathogenicity-	Mechanism	Clinical features

	associated genes or factors		
Enteroinvasive *E. coli*	140MDa plasmid (pINV) chromosomal genes	Bacterial attachment and invasion of colonic enterocytes via endocytosis, multiplication causing host-cell death and inflammation, accompanied by necrosis and ulceration of large bowel	Ulceration of bowel, watery diarrhoea, dysenteric stools (bacillary dysentery)
Enterotoxigenic *E. coli*	Plasmid-encoded CFAs: CFA-I (rigid rod-like fimbriae), CFA-III (bundle-forming group), CFA-II and CFA-IV (flexible fimbriae) and type IV-related longus pili Labile toxins: LTI, LTII (plasmid encoded) Heat-stable toxins: STa, STb (plasmid	Colonization of surface of small-bowel mucosa (CFA I–IV) and production of enterotoxins LTI, LTII, STa, STb ADP ribosylation of G proteins →adenylate cylase activation →increased cAMP secretion →reduced Na absorption/Cl secretion →diarrhoea	Acute watery diarrhoea, usually without blood mucus or pus

	and transposon encoded)	Guanylate cyclase (G C-C) activation → increased cGMP secretion →chloride secretion and/or inhibition of NaCl absorption →diarrhoea	
Enteroaggregative *E. coli*	Plasmid (60MDa) encoded: Aggregative adherence fimbriae (AAF/I and AAF/II), transcriptional regulator (AggR) *E. coli* heat-stable-like toxin-1 (EAST-1) ShET1 is *Shigella* enterotoxin-1Plasmid-encoded toxin (Pet)	Adherence and colonization of intestinal mucosa facilitated by AAF/I and AAF/IIRelease of toxins and damage to host epithelial cells	Aggregative adherence (AA) phenotype Persistent diarrhoea
Diffusely adherent *E. coli*	Afa/Dr family adhesions (AIDA) EAST-1 *set* genes (enterotoxins) Possible TTSS with *esc*	DA phenotype facilitated by surface fimbriae, e.g. F1845 encoded via *daaC* gene, or by other related adhesions which are	Diffusely adherent (DA) Phenotype Watery diarrhoea, usually without blood

		plasmid or chromosomally encoded Events in pathogenesis remain unclear	
Enteropathogenic *E. coli*	50–70MDa plasmid (EAF) encodes: bundle-forming pilus (BFP), plasmid-encoded regulator (Per) and LEE-encoded regulator (ler) Chromosomal PAI (LEE) TTSS comprising: intimin (*eaeA*), secreted proteins; Tir, EspA, EspB, EspD, EspF, EspG and mitochondria-associated protein (MAP) EAST-1 Cytolethal distending toxin (CDT)	Localized adherence (LA) via BFP A/E histopathology; cytoskeletal rearrangement of host epithelial cells involving TTSS Intimate effacing adherence mediated by intimin Destruction of microvilli and interference with host-cell signaling cascades	A/E lesiones Acute diarrhoea
Enterohaemorrhagic *E. coli*	Large (60MDa) plasmid encodes: enterohaemolysin	A/E histopathology and intimate adherence	Bloody diarrhoea (hemorrhagic colitis)

Verocytotoxin-producing *E. coli*	(Ehx), LCT, EspP Chromosomal PAI (LEE) Chromosomal (prophage) encoded Shiga toxins (Stx)/verocytotoxin (VT) VT1, VT2 and VT2v	Similar to EPEC. Alternative adherence Mechanisms (besides *eae*) known. TTSS aids pathogenesis, toxins (Stx/VT) inhibit protein synthesis of host cells and mediate different pathological effects	Hemolyticuremic syndrome (HUS) TTP

A/E, attaching and effacing; CFA, colonization factor antigen; EAF, EPEC adherence factor; EAST, enteroaggregative heat-stable toxin; EPEC, enteropathogenic *E. coli*; Esp, *E. coli* secreted protein; LCT, large clostridial toxins; LEE, locus of enterocyte effacement; TTP, thrombotic thrombocytopenic purpura; TTSS, type III protein secretion system.

2.3. Antibiotic resistance

E. coli are resistant to many antibiotics those are effective against Gram-positive organisms. Antibiotic resistance is a growing problem (Johnson *et al.*, 2006). Antibiotic resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as Staphylococcus aureus. *E. coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species of bacteria (Salyers*et al.*, 2004).

2.3.1. Resistance patterns

Many recent studies have reported the resistance profiles of uropathogens to antimicrobial agents commonly used to treatUTI. Much of this in vitro data comes from laboratory-based surveys that often do not define the sex, age, clinical syndrome, or location (inpatient vs. outpatient) of the patients from whom the urine specimens are collected. Therefore, the reported rates of resistance may vary depending on whether the study sample consists primarily of outpatients with uncomplicated UTI or patients with complicated nosocomial UTI (Gupta *et al.*, 1999). In UTI, resistance patterns occur in two different ways. Firstly, there can be a higher prevalence of resistance to conventional antibiotics in the usual. Urinary pathogens such as *E. coli* and *Klebsiella* spp. Secondly, there can be an increased frequency of pathogens with greater intrinsic resistance to conventional antibiotics such as *Pseudomonas aeruginosa* (Nicolle, 2002). Since the management of UTI has traditionally been based on the fact that the spectrum of bacteria causing UTI and their resistance patterns are very predictable, empiric treatment with short course Trimethoprim-sulfamethoxazole (TMPSMX) has been the standard management approach (Gupta, 2002). Guidelines published in 1999 by the Infectious Diseases Society of America (IDSA) recommend TMP/SMX as first-line treatment for acute cystitis, however resistance to this agent is increasing. Although public health authorities have increasingly recommended narrow spectrum antibiotics for treating community-acquired urinary tract infection (CUTI) whenever possible, concerns about resistance have resulted in a burgeoning use of Fluoroquinolones (Hooton *et al.*, 2004). In the past few years, use of Fluoroquinolones in ambulatory care has dramatically increased, whereas use of TMP-SMX for UTI has been

decreased. In the past, antibiotic resistance has mostly been a problem in hospital acquired UTI. However, recent data suggest that antibiotic resistance in community acquired UTI is now also becoming an important consideration. In a 1998 surveillance study, ten tertiary-care hospital microbiology laboratories from across Canada collected bacterial isolates from two thousand consecutive outpatients with a UTI (Zhanel *et al*., 2000).

In the United States, resistance to TMP-SMX among *E. coli* isolates is increasing in context to community-acquired UTI now exceeds 20 percent in many parts of the country. The prevalence of such resistance is substantially higher in the West (22 percent) than in the Northeast (10 percent), with substantial variation from hospital to hospital. Resistance to TMP-SMX is generally associated with resistance to additional drugs. The resistance to TMP-SMX is noticed in the southern Europe, Israel and Bangladesh, where the prevalence of resistance is now 30 to 50 percent (Gupta *et al*., 2001). A greater proportion of isolates from both practices were resistant to Ampicillin, Amoxicillinclavulanic acid, Cefuroxime, Ceftazidime and Cotrimoxazole in 2003 when compared to 1999. With respect to *E. coli,* there were significant increases in prevalence of resistance to Cefuroxime and Amoxicillin-clavulanic acid. The overall resistance rate for Norfloxacin remained relatively low and was unchanged for *E coli.* Continued surveillance of uropathogen resistance trends is important and this information should be communicated to clinicians. The feasibility of using the Fluoroquinolones as a first line of therapy in urinary tract infection should be considered (Orrett and Davis, 2006).

2.4. Host defense mechanisms

Several defense mechanisms exist in order to prevent colonization and infection. The normal flora of the periurethral area forms a defense against the colonization of pathogenic bacteria. Alterations in this environment resulting from shifts in pH or estrogen levels or the use of antimicrobial agents may alter the makeup of the protective flora and also the ability of pathogenic bacteria to colonize (McRac *et al*., 2000). Urine itself possesses antibacterial activity with extremes in osmolarity, high urea concentrations, and low urinary pH. Prostatic fluid inhibits bacterial growth, while the presence of glucose can favour bacterial growth (Hooton et *al*., 1996). Constitutive mechanisms involved in the clearance of bacteria include mechanical factors such as urine flow and regular bladder emptying (Bates *et al*., 2004) but catherization can impair this defense and promote infection. A multitude of biochemical factors have been identified, including cytokines, immunomodulators, adhesion proteins, humoral immunity and antibodies. Bacterial adherence of epithelial cells elicits recruitment of polymorph nuclear cells (PMNs) and results in production of cytokines such as interleukin (IL)-1â, IL-6, IL-8, and tumor necrosis factor. Different urinary inhibitors of bacterial adherence are secreted and include Tamm-Horsfall protein, bladder mucopolysaccharide, low-molecular weight oligosaccharides, serum immunoglobulin A (sIgA) and lactoferrin (Hooton *et al*., 1996).

2.5. Virulence factorof *Escherichia coli*

2.5.1. Type 1 and P fimbriae

Type 1 fimbriae are characterized as having the ability to agglutinate chicken and guinea pig erythrocytes in the absence of D mannose (Kariyawasam and Nolan, 2009). They consist of a major protein, *Fim*A,

associated with ancillary proteins *Fim*F, *Fim*G, and the adhesion protein *Fim*H, encoded by the *fim* gene cluster (Orndorff and Falkow, 1984; Feria *et al*., 2001). This type of fimbria is common among Enterobacteriaceae; also several variants have been strongly associated with UPEC. Their role in infection is unclear although it has been suggested that they may be involved in the initial stages of colonizing in the upper respiratory tract (Simms and Mobley, 2008; Katouli, 2010).

2.5.2. Adhesion-encoding genes

The *pap* gene cluster consists of 11 genes encoding the main component of the pilus rod (*PapA*), which determines 11 different serogroups, and a terminally located adhesion, *PapG* (Brunder *et al*.,2001., Srimanote *et al*.,2002).

The S fimbriae are mannose-resistant adhesions, encoded by the *sfa* operon of uropathogenic *E. coli* (Stordeur *et al*., 2004). The presence of S fimbriae is also correlated with pathogenicity of *E. coli* in human meningitis and septicemia (Antao *et al*., 2009).

2.5.3. Alpha hemolysin (α-Hly) and cytotoxic necrotizing factor 1 (CNF-1)

Alpha hemolysin (α-Hly) and cytotoxic necrotizing factor 1 (CNF-1) are two well-known toxins of UPEC proven to have direct cytotoxicity to host tissues (Keane *et al*.,1987., De Rycke *et al*.,1987). HlyA is usually encoded by PAIs of UPEC together with or without CNF-1 (Davis *et al*., 2005). A CNF-1-positive UPEC strain caused greater acute inflammatory response of the bladder in a mouse model of ascending UTI than did its isogenic CNF-1 mutant (Mills *et al*., 2000).

2.5.4. Aerobactin

Aerobactin is a bacterial iron chelating agent (siderophore) (Neilands, 1995) found in *E. coli*. (Johnson *et al*., 1988) It is a virulence factor enabling *E. coli* to sequester iron in iron-poor environments such as the urinary tract. (Meyrier, 1999).

Aerobactin is biosynthesized by the oxidation of lysine, catalyzed by the enzyme aerobactin synthase, which is then coupled to citric acid. The gene for this enzyme is found in the aerobactin operon, which is roughly 8 kilobases long and contains 5 or more genes in total (de Lorenzo *et al*., 1986). *Yersinia pestis* contains genes relating to aerobactin, but they have been inactivated by a frame shift mutation, thus *Y. pestis* is no longer able to synthesize aerobactin (Forman *et al*., 2007).

2.6. Laboratorymethodsforisolationanddetectionof pathogenic*E. coli*

Conventional scheme

Whilst most strains of *E. coli* grow well on a range of microbiological culture media, the growth and isolation of some pathogenic strains requires specific methodology. Strains of pathogenic *E. coli* can be phenotypically identical to commensal *E. coli* strains, whereas others may give rise to atypical reactions with particular biochemical tests which aid the identification of *E. coli*. Rapid lactose fermentation remains a key diagnostic feature of media used for the initial isolation or subsequent confirmation of *E. coli*. MacConkey agar and *E. coli* broth are widely used for the initial isolation and confirmation of suspect *E. coli*, respectively(Stephen and peter, 2006). Strains of *E. coli* are commonly

distinguished from other fecal coliforms by their ability to grow and produce gas from lactose at 44C and indole production from tryptophan. However, these two tests are not always exclusive to *E. coli* as other bacteria, e.g. *Klebsiella*, can give rise to false-positive results. Furthermore, strains of EIEC often ferment lactose slowly or not at all, which together with the absence of indole production and synthesis of lysine decarboxylase can mean that they are not recognized as *E. coli* (Stephen and peter, 2006).

The presumptive identification of *E. coli* has been improved by the introduction of chromogenic media that provide better diagnostic characteristics mediated by specific enzyme activity which yields colonies of a distinct color. Most chromogenic substrates used in *E. coli* specific media rely upon the activity of GUD which is prevalent in approximately 95% of *E. coli* strains. A notable exception, however, is *E. coli* O157:H7, which is largely GUD negative (Stephen and peter, 2006). Another common enzyme exploited in chromogenic media is D-galactosidase which is responsible for lactose fermentation and common in coliform bacteria, including *E. coli*. Some media contain individual chromogenic substrates to enable specific identification of the target organism, whereas others contain more than one substrate, which enables a differential count or presumptive identification to be made (Stephen and peter, 2006). Fluorogenic substrates that follow the same principle are also available, although they are now less popular because of the requirement to observe plates under long-wave UV light and the problems caused by diffusion of fluorescence through the medium. Lactose fermentation remains a useful diagnostic feature of media for the isolation of urinary pathogens, including *E. coli*. A good example of such a medium is cysteine lactose

electrolyte-deficient (CLED) agar which is used for routine diagnostic urinary bacteriology. This medium is recommended because it is reported that 1.5% of *E. coli* isolated from urine require cysteine and that subsequent broths used for their identification will require cysteine supplementation (McIver and Tapsall, 1990). The non-selective medium CLED supports the growth of a wide range of urinary pathogens whilst preventing swarming of *Proteus* spp. Lactose-fermenting organisms, including *E. coli*, lower the pH of the medium, which turns from green to yellow. The development of chromogenic media combining the basal CLED medium with various chromogenic substrates has the potential to improve presumptive identification of urinary isolates (Fallon *et al.*, 2003).

The correlation between specific O and H antigens with different pathotypes of *E. coli* has led to serotyping being used for identification purposes. Whilst this remains useful for certain pathotypes associated with infection, especially those that comprise limited serotypes and strains belonging to distinct clonal lineages, this becomes less reliable when the pathogenicity-associated genes are located on mobile genetic elements. Consequently, there is no longer a clear distinction between certain pathotypes of *E. coli* based on serotyping. Detection of specific antigens or toxins associated with a particular *E. coli* pathotype using appropriate immunological methods such as ELISA can improve detection and identification of these strains. This approach has been used to confirm ETEC colonies using a GM1 ganglioside ELISA to detect LT and ST, which compared favorably against a gene probe (Sommerfelt *et al.*, 1988). Numerous commercial assays are available for the detection of the somatic O antigen of *E. coli* O157 and also for the detection of VT from culture

supernatants or directly from sample enrichments, thus enabling detection of all VTEC in clinical samples and foods (Bettelheim and Beutin, 2003).

Molecular scheme

Although phenotypic tests remain useful for the presumptive identification of pathogenic *E. coli*, the introduction of rapid molecular based technologies has revolutionized clinical diagnosis. Detection and confirmation of specific DNA sequences associated with known pathogenicity-associated genes or conserved regions unique to a particular pathotype can be used to aid confirmation of the presence or identity of these bacteria. DNA probes and techniques such as polymerase chain reaction (PCR) can be applied directly to clinical samples and foods. Alternatively, PCR can be applied directly to suspect colonies to confirm the presence of specific gene sequences. For the detection of ETEC, this has included using non-radioactively labeled oligonucleotide DNA probes and PCR targeted against the genes encoding LT and ST (Yavzori *et al*., 1998) and EAST-1 (Yamamoto and Echeverria, 1996). Popular targets for probe and PCR-based detection of EPEC strains include the EAF plasmid (Franke *et al*., 1994) and the gene encoding BFP (*bfpA*) (Gunzburg and Riley, 1995). Demonstrating AA pattern in the HEp-2 assay is used to confirm the presence of EAggEC, but this has been improved by the development of an EAggEC-specific probe (Baudry *et al*., 1990). Detection of EAggEC and DAEC strains has been improved by using PCR primers targeted against various plasmid and chromosomally encoded genes associated with adherence and colonization.

Detection of EHEC strains, particularly *E. coli* O157:H7 has received much attention recently owing to the risks posed by these bacteria and the severity of the infection they cause. Unlike typical *E. coli*, including other

VTEC strains, most O157:H7 strains share the inability to ferment sorbitol in 24 h, which is exploited in selective plating media, the most commonly used medium being sorbitol MacConkey agar (SMAC).Since it was described for the differentiation of *E. coli* O157:H7 from other *E. coli* in clinical samples (March and Ratnam, 1986), SMAC and modified versions of this medium have been universally adopted as the medium of choice for the isolation of *E. coli* O157. A limitation of relying solely on the lack of sorbitol fermentation for the isolation of *E. coli* O157 is the existence of sorbitol-fermenting (SF) strains (Fratamico, Buchanan and Cooke, 1993) and O157 strains that display both SF and GUD activity (Gunzer *et al*., 1992). Alternatives to SMAC for O157 isolation include chromogenic media (Restaino *et al*., 1999),including some that allow isolation and recognition of other VTEC (Bettelheim, 1998) and blood agar containing washed sheep red cells, which allows recognition of Ehx production (Beutin *etal*., 1989).

Detection of nucleotide sequences related to the toxin (*stx/vtx*) genes including *vtx2* variants using the PCR technique has become a popular method for detecting VTEC in foods and clinical samples (Lin *et al*., 1993). DNA probes have also been developed for the confirmation of suspect isolates (Samadpour and Liston, 1994). Alternative targets for DNA probes and PCR include the hemolysin (*HlyA*) gene (Lehmacher *et al*., 1998), the *E. coli* attaching and effacing (*eae*) gene (Louie *et al*., 1994), the GUD (*uidA*) gene (Feng, 1993), the 60-MDa plasmid found in O157:H7and other VTEC (Levine *et al*., 1987) and the O157 *fliC* (flagellin), gene (Gannon *et al*., 1997).

Multiplex PCR has been used to simultaneously detect EHEC, EPEC and ETEC in fecal samples from patients with watery diarrhoea, HC and HUS

using primers targeted against *eae, bfp*, *stx1*, *stx2*, *lt* and *st* (Vidal *etal*., 2004).

3. Materials and Methods

3.1. Type of study

This study was descriptive cross sectional laboratory based study.

3.2. Study area and Sample collection

The study was carried out in Khartoum Stateduring the period from March to May, 2017. A total of 100 *E. coli*samples (50 urine, 50 diarrhea)were obtained from different Hospitals (Omdurman military hospital, Police hospital, Soba hospital, Bahary hospital) in Khartoum state, which previously isolated from patients with diarrhea and urinary tract infection.

3.3. Identification scheme

3.3.1. Conventional methods

Growth examination

Standard amount of urine and diarrhea specimen from each patients were inoculated separately into this suitable media;

MacConkey agar: For isolation of Gram negative enteric bacteria and to differentiate lactose fermenting organisms from non-lactose fermenting organisms.

CLED agar: It is a non-selecting differential plating medium for growth and enumeration of urinary tract microorganism. Here indicator dye, (bromothymol blue) is used to differentiate lactose fermenting from non-lactose fermenting bacteria. Electrolyte deficient prevents swarming of *Proteus.*

Nutrientagar

This media was used for purification andshort preservation of organisms.

(Collee *et al.*, 1996).

Colony morphology

MacConkey agar: Produce smooth pink colony. CLED agar: Smooth, circular, 1.5mm diameter, yellow opaque colony. Blood agar: Rounded colonies of 1.4 mm diameter with or without hemolysis (Collee *et al.*, 1996).

Gram Stain

The procedure was carried out according toCheesbrough,(2006) 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 Bunzen 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 Saffranin 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.

Biochemical tests

Using sterile straight loop a colony was touched and inoculated on (Kligler iron agar, tryptophan peptone water, semisolid media, Simmon's citrate agar, Christensen's urea agar) (HIMEDIA, India), and then incubated at 37ºC overnight, then interpreted after adding kovac's reagent to tryptophan

peptone water medium, All *E.coli* isolates were glucose and lactose fermenters (gave yellow butt and yellow slope on KIA), and they give positive indole test (red ring), and they were motile (diffuse growth on the semisolid media).And they not grow on simmon's citrate agar and gave negative urease test (Collee *et al.*, 1996).

Susceptibility testing

Susceptibility pattern was done by disk diffusion method. All the isolated organisms were put into appropriate media for antibiotic susceptibility test. Disc diffusion tests were performed and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2007). 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 took out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at 37 degree for 24 hours. On the next day, 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) and sensitive (S), according to the reference zone of inhibition of particular antibiotic (NCCLS, 2001).

3.3.2.Molecular characterizations

DNA Extraction for Polymerase Chain Reaction

Genomic DNA (templates) for PCR amplification were extracted from overnight growth of bacterial isolates on nutrient agar (several colony) suspended in 1000 µL of sterile deionized water, and boiled for 15

minutes. After centrifugation of the boiled samples at 14000 g for 10 minutes,supernatant was stored at -20°C as a template DNA stock(Yamamoto *et al*., 1995).

Gel electrophoresis of extracted DNA

The purity of the extracted DNA was determined by running the DNA sample on 1.5% gel agarose (Sambrook *et al*., 1989).

Preparation of 10 X TBE buffer

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.

Preparation of 1X TBE buffer

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

Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

Preparation of agarose gel

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, 5 µl of (10mg\ml) Ethidium bromides 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(Jalali *et al*., 2015).

Polymerase Chain Reaction Amplification

The primers were synthesized by (Macrogen, Korea).Specific primers were used to amplify the *fimH, pap, sfa, hly* and *aer* genes as indicated in table (3).

PCR was done by multiplex PCR, amplification was done using TECHNE® Ltd peltier thermal cycler (Germany), DNA amplifies was done using Maxime PCR Premix kit (iNtRON, Korea),The PCR assay was carried out in a total volume of 25 µL of mixture containing 2 µL Maxime PCR Premix containing 1X PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, and 1 U Taq DNA polymerase, 0.5 µL of each of the virulence gene-specific primers (5 µL), 2 µL of template DNA and 16 µL of WFI (water for injection). The amplification conditions included three steps: heating at 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 30sec; and the final extension at 72°C for 7 min(Jalali *et al*., 2015).

Table (3) Primers used for detection of virulence genes in UPEC strains (Jalali *et al*., 2015).

Identified Gene	Primer	Primers Sequence, (5-3)	Product size (bp)
papE/F	*Pap3* *pap4*	GCAACAGCAACGCTGGTTGCATC AT AGAGAGAGCCACTCTTATACGG	336

		ACA	
fimH	*fim1* *fim2*	GAGAAGAGGTTTGATTTAACTTATTG AGAGCCGCTGTAGAACTGAGG	508
sfaD/E	*sfa1* *sfa2*	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
aer	*aer1* *aer2*	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602
hlyA	*hly1* *hly2*	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177

Visualization of the DNA products

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface,10 µl of PCR products from each samples was added to wells of electrophoreses, 5 µl of100-bp DNA ladder (iNtRON, Korea),was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (100 V, 500 mA, UK). 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 transilluminater and photographed (Uvitec – UK), (Jalali *et al*., 2015).

3.3.3.Data analysis

Data was analyzed by using Statistical Package for Social Science Program (SPSS) version (11.5), by using Chi square *p*value less than 0.5 was consider significant for the association between variables (IBM, 2012).

4. RESULTS

A total of 100 patients, 50 patients suffering of UTIs, 50 patients suffering of diarrhea who attending Khartoum during March to May 2017, were enrolled in this study.

4.1. The association between the presence of UPEC virulence genes andAge group

Most of study population were females 57 (57%) 42 suffering of UTIs 15 suffering of diarrhea compared with males 43 (43%) 8 suffering of UTIs 35 suffering of diarrhea, their age ranged from 10 to 50 with mean 29 years, and most of them were in the 21 – 30 years as indicated in Table (4), Figure (1).

Table (4)Association between the presence of UPEC virulence genes and Age group

Genes	Age group 10-20	Age group 21-30	Age group 31-40	Age group 41-50	P-value
fim	4	16	13	8	0.167
pap	4	11	8	10	0.220
sfa	4	6	4	1	0.508
aer	11	14	11	10	0.716
hly	3	3	7	4	0.428

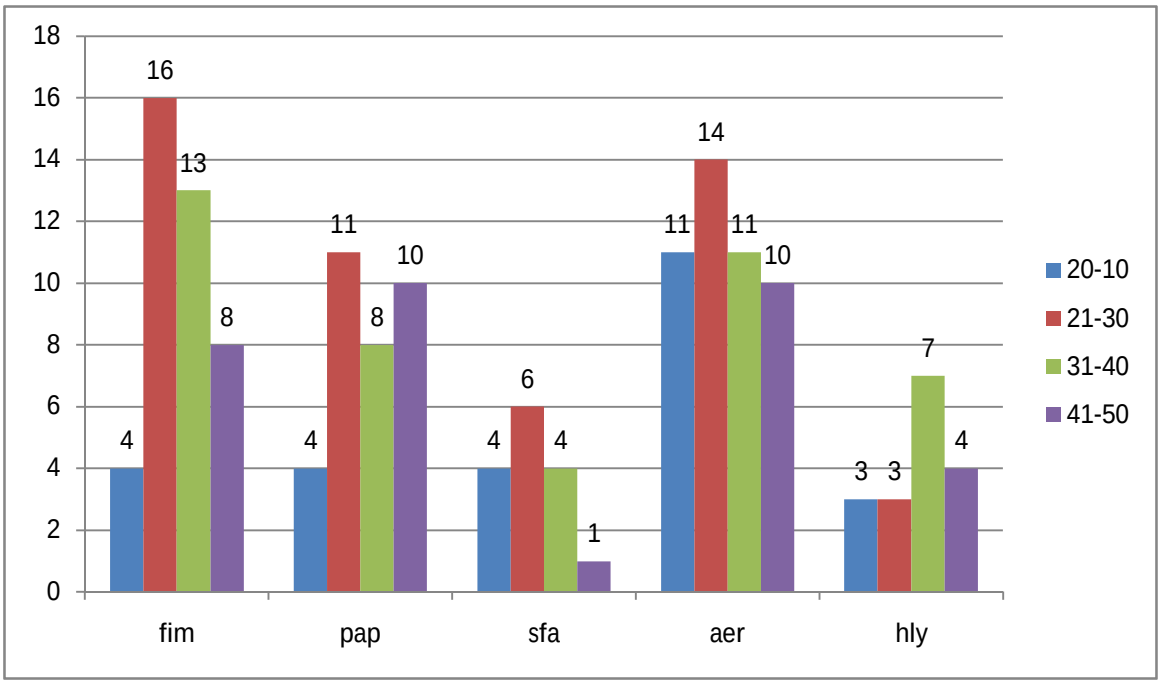

Figure (1) Age group of enrolled patients and relationship with UPEC virulence genes

4.1.2. Association between the presence of UPEC virulence genes and gender

The overall results revealed that total of 43 male, 57 female, 22 (38%) females positive for *pap* gene. 30 (52%) females positive for *fim*gene. 12 (21%) females positive for *sfa*gene. 25 (44%) females positive for *aer* gene. 11 (19%) females positive for *hly* gene indicated in Table (5), Figure (2).

Table (5)Association between the presence of UPECvirulence genes and gender

Genes	*pap*		*fim*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Male	11	32	11	32	3	40	21	22	6	37	43
Female	22	35	30	27	12	45	25	32	11	46	57
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.171		0.006		0.051		0.621		0.481		

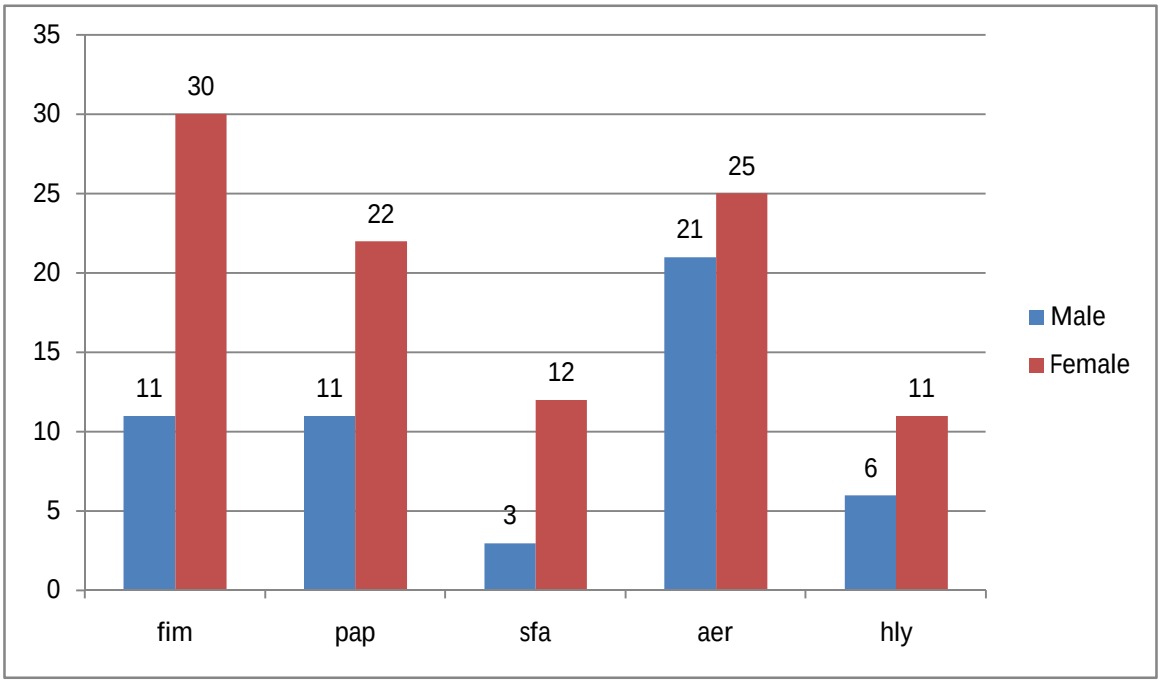

Figure (2) Association between presence of UPEC virulencegenes and gender

4.1.3. The association between the presence of UPEC virulence genes andSamples

The overall results revealed that total of 50 urine sample, 50 diarrhea sample, 46 (46%) positive for *aer* gene Figure (5), 32 positive in diarrhea. 41 (41%) positive for *fim*gene, 33 positive in UTIs. 33 (33%) positive for *pap*gene, 24 positive in UTIs. 17 (17%) positive for *hly* gene, Figure (4), 14 positive in UTIs. 15 (15%) positive for *sfa* gene, 15 positive in UTIs as indicated in Table (6), Figure (3).

Table (6)Presence of UPEC virulence genes in urine and diarrhea samples

Genes	***pap***		***fim***		***sfa***		***aer***		***hly***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Urine	24	26	33	17	15	35	14	36	14	36	50
Diarrhoea	9	41	8	42	0	50	32	18	3	47	50
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.001		0.000		0.000		0.000		0.003		

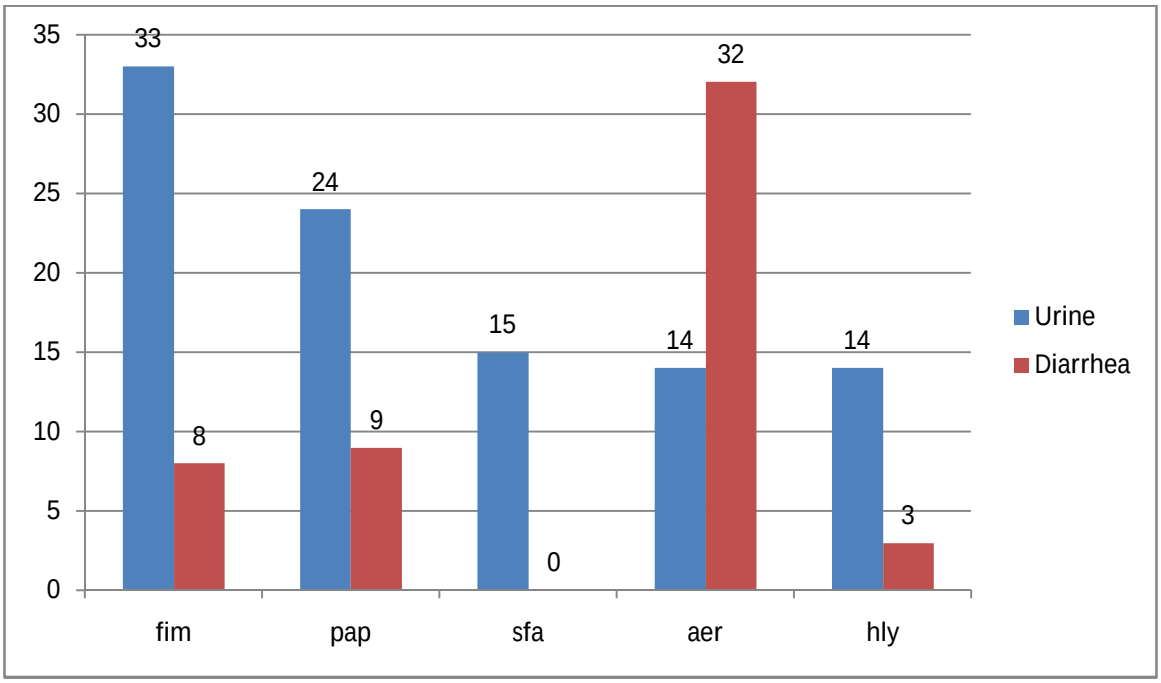

Figure (3) Presence of UPEC virulence genes in urine and diarrhea samples

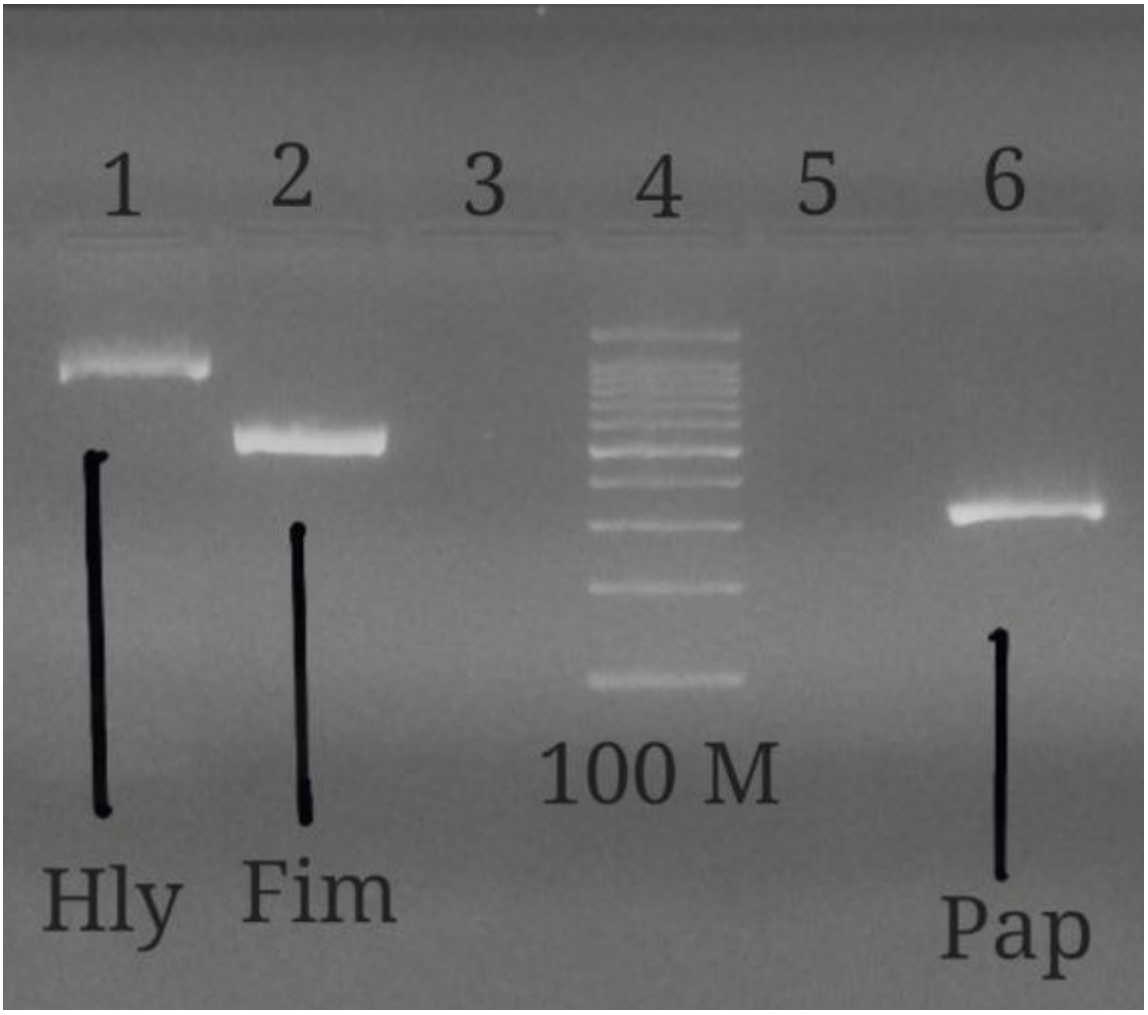

Figure (4) Agarose gel electrophoresis of multiplex PCR product 1= positive *hly* gene 2: positive *fim* gene 3, 5: negative samples 4: 100 bp ladder 6: positive *pap* gene.

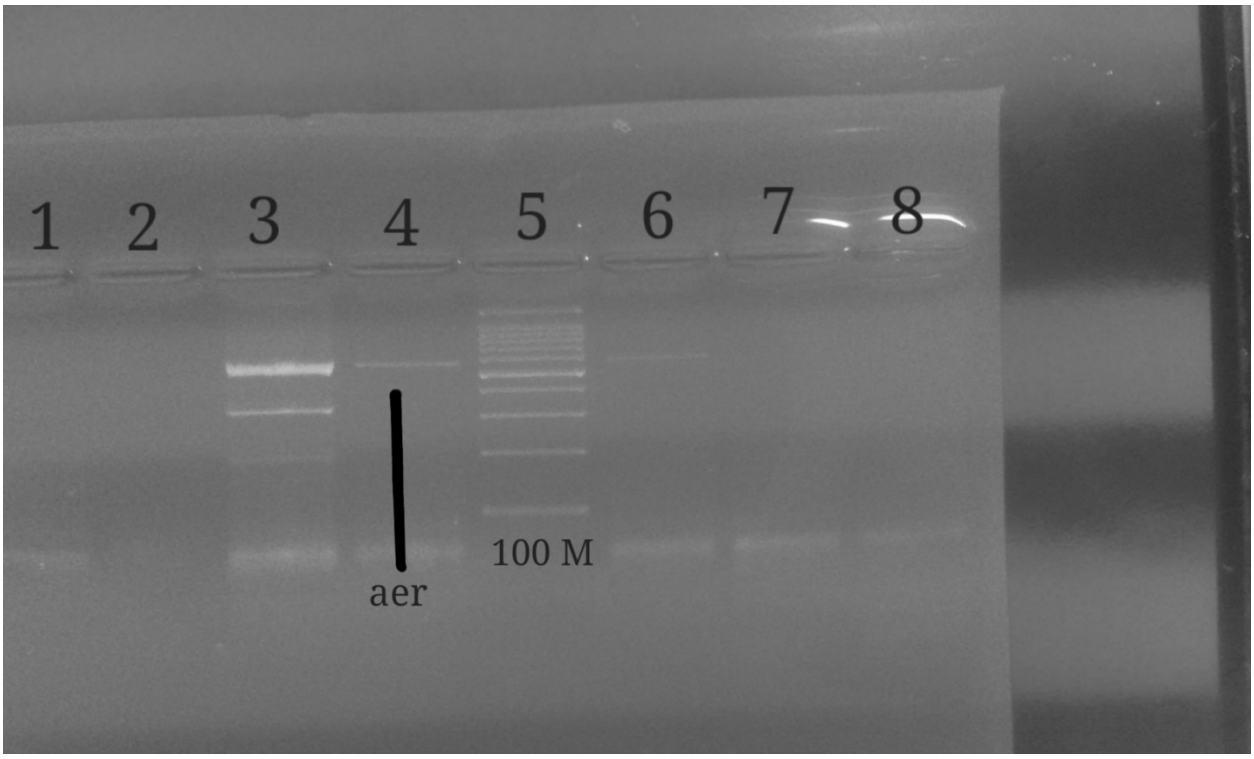

Figure (5) agarose gel electrophoresis of multiplex PCR product 1,2,7,8: negative samples3: positive*pap, aer* genes4: positive *aer* gene 5: 100 bp ladder 6: positive *aer* gene

4.1.4. The association between the presence of UPEC virulence genes andAntibiotic

Four broad spectrum antibiotic discs are used to perform antimicrobial susceptibility test, Gentamycin, Amikacin, Ciprofloxacin, Co-trimoxazole.

Gentamicin

The overall results revealed that total of 55 sample sensitive for Gentamicin there was no significant association between the presence of (*fim, sfa, aer, hly*) genes to gentamicin antibiotic (p-value 0.526, 0.673, 0.354, 0.073) but there is significant strong association between the presence of *pap* gene and resistance to gentamicin (p-value 0.000),21 (38%) sensitive for *fim* gene, 10 (18%) sensitive for *pap* gene, 9 (16%) sensitive for *sfa* gene, 23 (42%) sensitive for *aer* gene, 6 (11%) sensitive for *hly* gene, as indicated in Table (7), Figure (6).

Table (7)Association between presence of UPECvirulencegenes and susceptibility to Gentamicin

Genes	*pap*		*fim*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	10	45	21	34	9	46	23	32	6	49	55
Resistant	23	22	20	25	6	39	23	22	11	34	45
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.000		0.526		0.673		0.354		0.073		

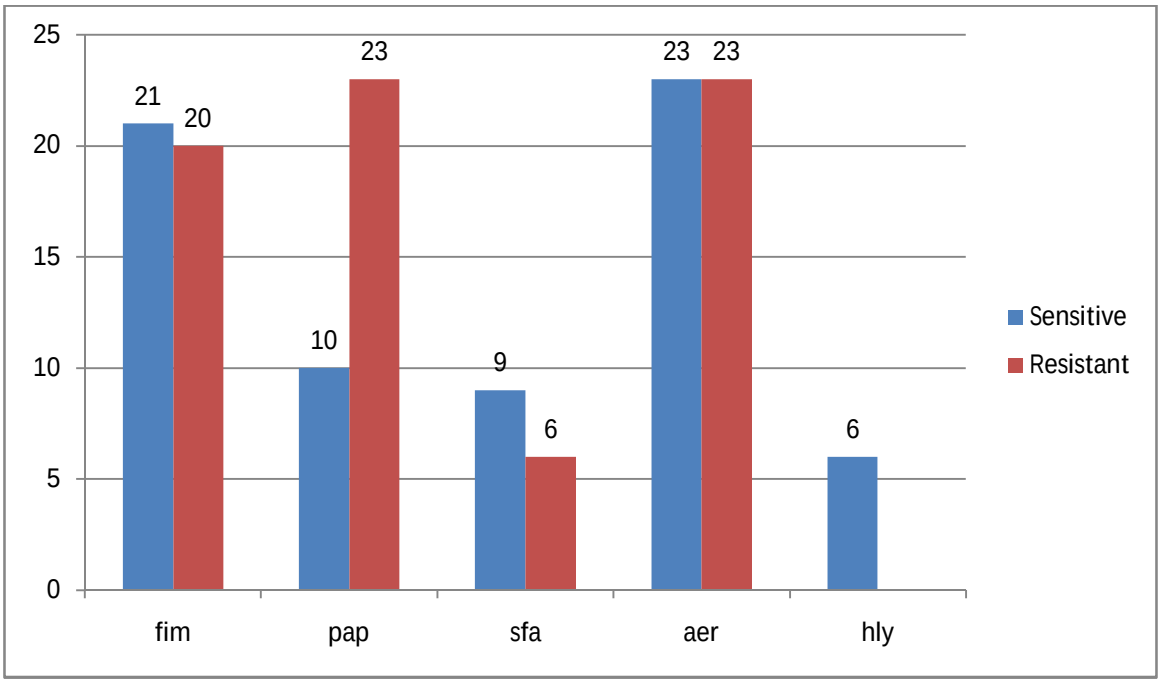

Figure (6) Association between presence of UPEC virulencegenes and Gentamicin

Amikacin

The overall results revealed that total of 96 sample sensitive for Amikacin there was no significant association between the presence of (*fim*, *sfa*, *aer*, *pap*) genes to Amikacin antibiotic (p-value 0.158, 0.568, 0.235, 0.068) but there is significant strong association between the presence of (*hly*) gene and resistance to Amikacin (p-value 0.002), 38 (40%) sensitive for *fim*gene, 30 (31%) sensitive for *pap* gene, 14 (15%) sensitive for *sfa*gene, 43 (45%) sensitive for *aer* gene, 14 (15%) sensitive for *hly* gene, as indicated in Table (8), Figure (7).

Table (8)the association between presence of UPEC virulence genes and Amikacin

Genes	*pap*		*fim*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	30	66	38	58	14	82	43	53	14	82	96
Resistant	3	1	3	1	1	3	3	1	3	1	4
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.068		0.158		0.568		0.235		0.002		

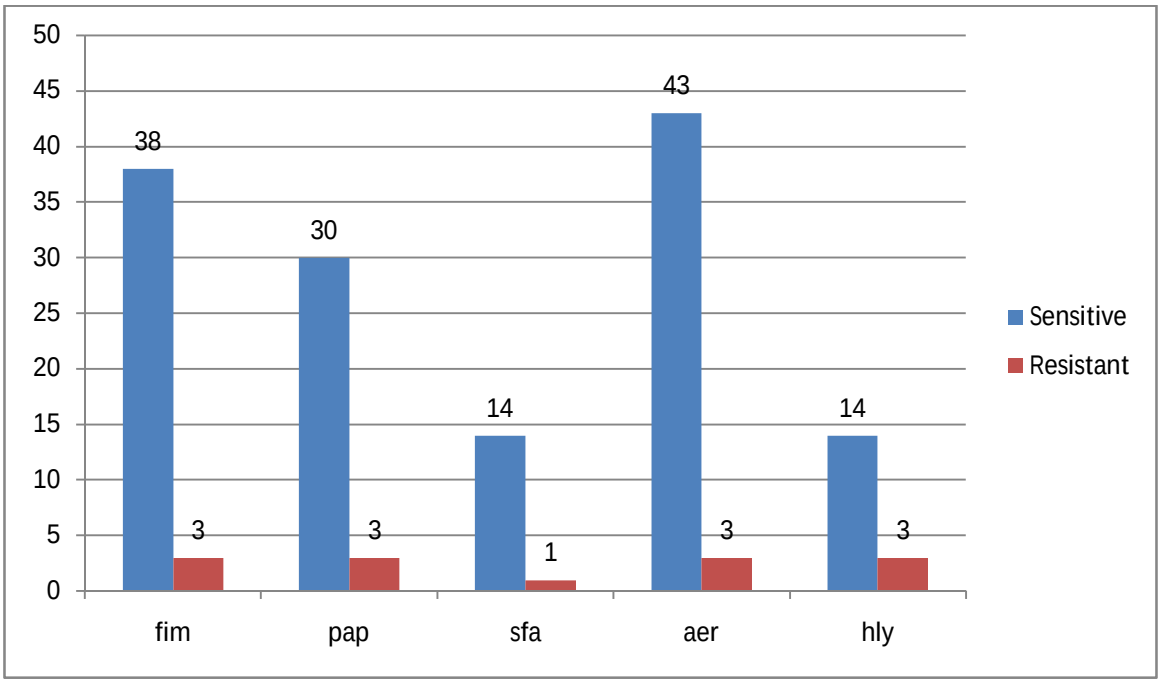

Figure (7) Result theassociation between presence of UPEC virulence genes and Amikacin

Ciprofloxacin

The overall results revealed that total of 57 sample sensitive for Ciprofloxacin there was no significant association between the presence of

(*fim*, *aer*,) genes to Ciprofloxacin antibiotic (p-value 0.330, 0.192) but there is significant strong association between the presence of (*pap*, *sfa*, *hly*) gene and resistance to Ciprofloxacin (p-value 0.039, 0.045, 0.002), 21 (37%) sensitive for *fim* gene, 14 (25%) sensitive for *pap* gene, 5 (9%) sensitive for *sfa* gene, 23 (40%) sensitive for *aer* gene, 4 (7%) sensitive for *hly* gene, as indicated in Table (9), Figure (8).

Table (9)the association between presence of UPECvirulence genes and Ciprofloxacin

Genes	*pap*		*fim*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	14	43	21	36	5	52	23	34	4	53	57
Resistant	19	24	20	23	10	33	23	20	13	30	43
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.039		0.330		0.045		0.192		0.002		

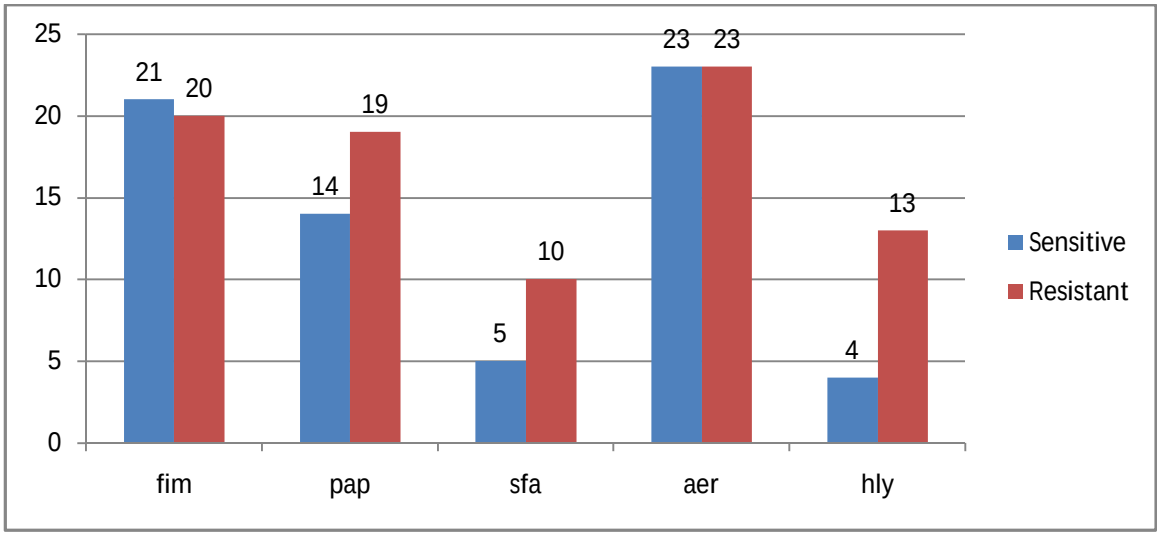

Figure (8) the association between presence of UPECvirulence genes and Ciprofloxacin

Co-trimoxazole

The overall results revealed that total of 63 sample sensitive for Co-trimoxazole there was no significant association between the presence of (*sfa*, *aer*,) genes to Co-trimoxazole antibiotic (p-value 0.155, 0.993) but there is significant strong association between the presence of (*fim, pap*, *hly*) gene and resistance to Co-trimoxazole (p-value 0.042, 0.035, 0.041), 21 (33%) sensitive for *fim* gene, 16 (25%) sensitive for *pap*gene, 7 (11%) sensitive for *sfa* gene, 29 (46%) sensitive for *aer* gene, 7 (11%) sensitive for *hly* gene, as indicated in Table (11), Figure (9).

Table (10)the association between presence of UPECvirulence genes and Co-trimoxazole

Genes	***pap***		***fim***		***sfa***		***aer***		***hly***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	16	47	21	42	7	56	29	34	7	56	63
Resistant	17	20	20	17	8	29	17	20	10	27	37
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.035		0.042		0.155		0.993		0.041		

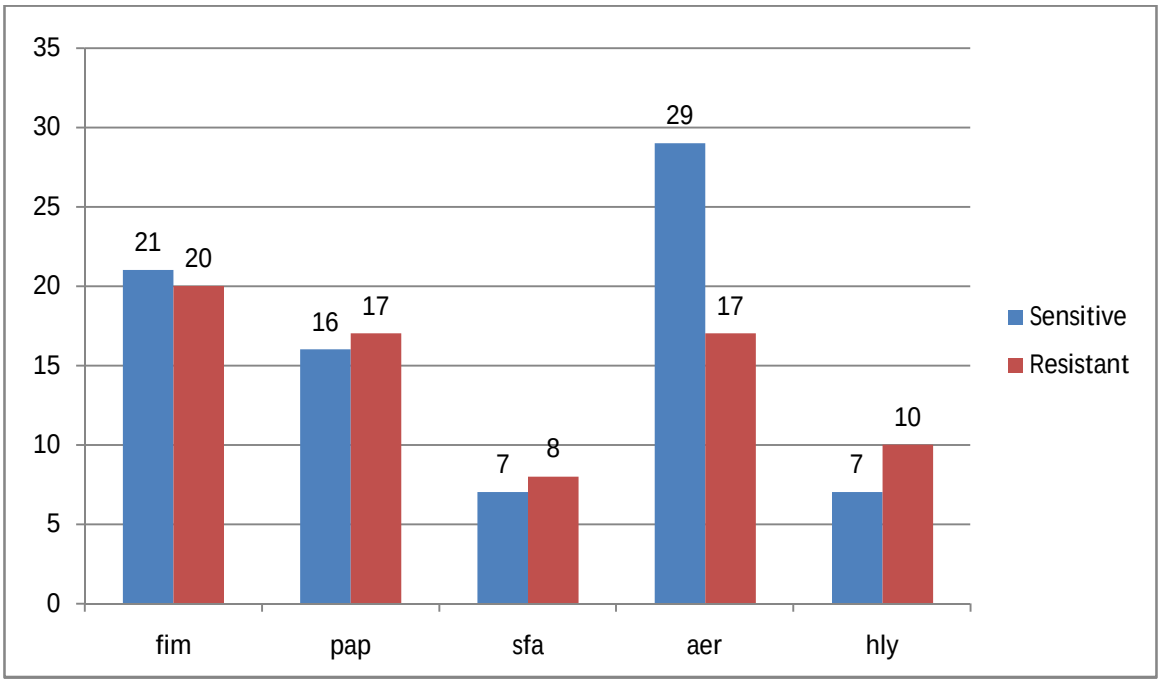

Figure (9) the association between presence of UPECvirulence genes and Co-trimoxazole

5. Discussion

Theresults of the present study showed higher frequency of *fimH* compared to the rest of the genes (66%), which may indicated an essential role of the virulence genes in *Escherichia coli* causing UTI among Sudanese patients. These results agree with published reports, which emphasize the predominance of fimbriae type 1 among the UPEC strains, also this result is not far fromJalali *et al*.(2015), who found73%*fimH* positive,and Tarchouna *et al*.(2013),who found 68%*fimH* positive,andUsein*et al*.(2001),who found 86%*fimH* positive among their study subjects.Fimbriae-mediated adherence is important for the virulence of *E. coli* in the urinary tract, Uropathogenic *E. coli*strains may express a variety of fimbrial adherence factors, such as P, S, Dr, and type 1 fimbriae. It has been established that P fimbriae enhance the virulence of uropathogenic strains through specific adherence and increased induction of mucosal inflammation, but the role of type 1 fimbriae in virulence remains undefined (Connell*et al*., 1996). We found significant association (p-value 0.006) between *fim* gene and gender; this association may be duo to difference in anatomical structure of urinary tract between male and female(Hickling *et al*., 2015).

Our results confirmed the existence of*aer* gene in diarrheal isolates, this findingswas totally agreed withOswald*et al*.(1991),who foundthat aer gene was positive in 70%of diarrheal samples, and Micenková *et al*.(2014), who found*aer* gene was positive in 68%. Low frequency of aer gene may be attributed to the deficiency of iron concentration within gastrointestinal tract while iron is responsible for microbial metabolism. Most *E. coli* can increase access to iron by excreting siderophores such as enterobactin,

which have a very strong affinity for Fe^{3+}. A smaller proportion of isolates can generate up to 3 additional siderophores linked with pathogenesis; aerobactin, salmochelin, and yersiniabactin (Meyrier, 1999).

The result show 24 (48%) *pap* gene positive in 50 urine isolate and this result is not far from Jalali *et al*. (2015), who found 46% *pap* positive gene, and Tarchouna *et al*. (2013), who found 41% *pap* positive gene. *Pap* (pyelonephritis associated pili), play an important role in the pathophysiology of pyelonephritis caused by *E. coli*.(Tarchouna *et al*., 2013). Uropathogenic *E. coli* (UPEC) utilize P fimbria (pyelonephritis-associated pili) to bind urinary tract endothelial cells and colonize the bladder. These adhesions specifically bind D-galactose- D-galactosemoieties on the P fimbria group antigen of erythrocytes and uroepithelial cells (Todar, 2007).

The result show 14 (28%) *hly* gene positive in 50 urine isolate and this percentage is less than result of Jalali *et al*. (2015), who found (47%) *hly* positive gene. The presence of hemolysin was related to tissue damage. Prevalence of these genes is different according to the phylogenetic groups, clinical conditions of host and geographical localization (Oliveira *et al*., 2011), (Blanco*et al*., 1997), although a large variation in gene frequencies has also been observed (Abe *et al*., 2008).

In this study by using PCR shown that the frequency of adhesions of UPEC strains, *fimH* and *pap* were more common and *hly, aer, sfa* similar to some studies and lower than some, Karimian *et al*. (2012), Oliveira *et al*.(2011).

The patients who had *fimH,* probably suffered from cystitis and descending infection or at least they are in such a field there was an

infection. On the contrary the patients who had *pap*, probably suffered from pyelonephritis and ascending infection or at least they are in such a field there was an infection. In patients who had also *sfa*, *hly* or *aer*, it is likely that there is already a primary sepsis. In the case of UPEC strains that did not have any virulence factors, can be said, all of them were have been related to normal flora of the gastrointestinal tract or may be asymptomatic bacteriuria (ABU) (Jalali *et al*., 2015).

There was 18 samples were negative for UPEC virulence genes 12 of them were in diarrheal samples and 6 were in urine samples. These negative isolates may be are normal flora, becausemost of them were also sensitive to all antibiotics used in this study. In addition to many virulence determinants contribute to the pathogenicity of *E. coli* in UTI,they are also the products of different genes which can be detected by PCR. However there is always the possibility of mutation at the level of the corresponding gene, leading to the absence of its detection. Therefore a positive PCR shows the presence of the virulence genes but a negative PCR does not point the absence of the corresponding operon (Tarchouna *et al*., 2013).

The present study showed there was a higher frequency of sensitivity to Amikacin (96%), because Amikacin does not used widely, due to it is high nephrotoxicity (Cho*et al*., 2016).

The present study result showed that the UPEC strains isolated in Sudan have a different virulence profile compared with other studies and it seems that the virulence of UPEC strains depends on the regional geography and climate. It is believed that the epidemiology and prevalence of UPEC strains virulence factors among Sudanese UTI patients are different from other countries. Perhaps some factors such as customs, food diets, public

health, and even methods of sampling have great rules in prevalence of virulence genes in UPEC strains (Jalali *et al.*, 2015).

6.1. Conclusion

The study concluded that:

- Fimbriae type 1 gene is highly prevalent among urinary tract infected patients, and aerobactin gene is highly prevalent among diarrheal patients.
- Fimbriae type 1 gene is highly prevalent among female, and aerobactin gene is highly prevalent among male.
- There was association between*fim* gene and (gender, sample and co-trimoxazole resistance), *pap* gene and (sample, gentamycin resistance, ciprofloxacin resistance and co-trimoxazole resistance), *sfa* gene and (sample and ciprofloxacin resistance), *aer* gene and (sample), *hly* gene and (sample, Amikacin resistance, ciprofloxacin resistance and co-trimoxazole resistance).
- Multiplex PCR was satisfactory for detection UPCE virulence genes.

6.2. Recommendations

- Further studies are needed to identify othervirulence factors of *Escherichia coli*responsible for UTI, and to determine the physiopathology of these infections to find possible prevention measures.
- Large sample size is recommended to identify theprevalence of UPEC virulence genes.
- Determination of antimicrobial resistant genesin association with virulence genes is a great importance.

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Appendix I – questionnaire

Sudan University of Science and Technology

College of Graduate studies

Genotyping the Virulence Factors of Uropathogenic *Escherichiacoli* isolates from diarrheic and urinary tract infection patients by multiplex PCR in Khartoum State

By: Husam Eldin Mohamed Hassan Musa

Supervised by: Hisham Nouraldayem Altayeb Mohammed

Name…….. Date:……

Index number: ……

Age:…… Gender:……

Symptoms:

1-fever …….. 2- back pain…….

3-burning sensation ……. 4- headache……..

5- diarrhea …… 6- vomiting……

Any treatment received……..

Previous diagnosis of UTI or diarrhea ……..

Culture result ……..

Sensitivity result……..

Signature:……..

Table (11) Biochemical reactions of most strains of *E. coli*

The test	The result
Motility	+ve
Indole	+ve
Citrate	-ve
Urease	-ve
H2S production	-ve
Lysine decarboxylase	+ve
Urine nitrite test	+ve

Appendix II

Reagents and Stains

Gram Stain (Cheesebrough, 2000)

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 (Hi Media)

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 (Hi Media)

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

E. coli 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).

Culture media

Preparation of Media (Chemie, 2014)

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.

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.

Appendix III

Figure (1) Microcenterfuge device

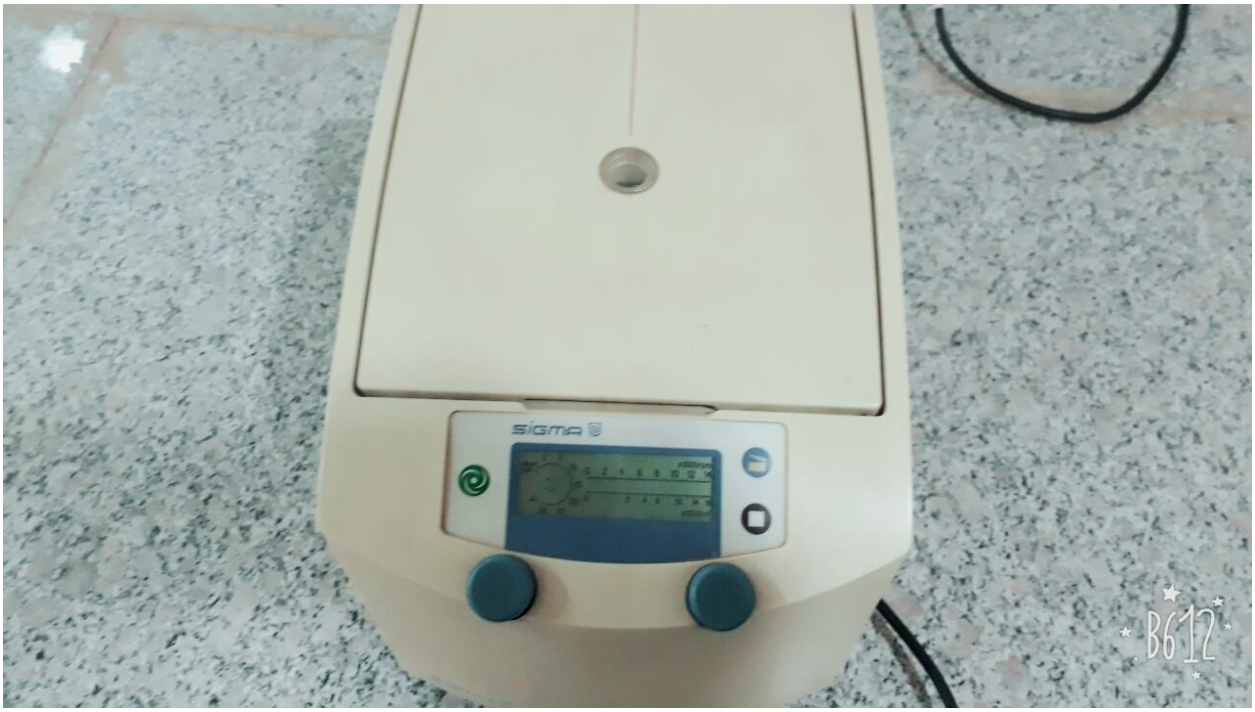

Figure (2) Thermocycle device

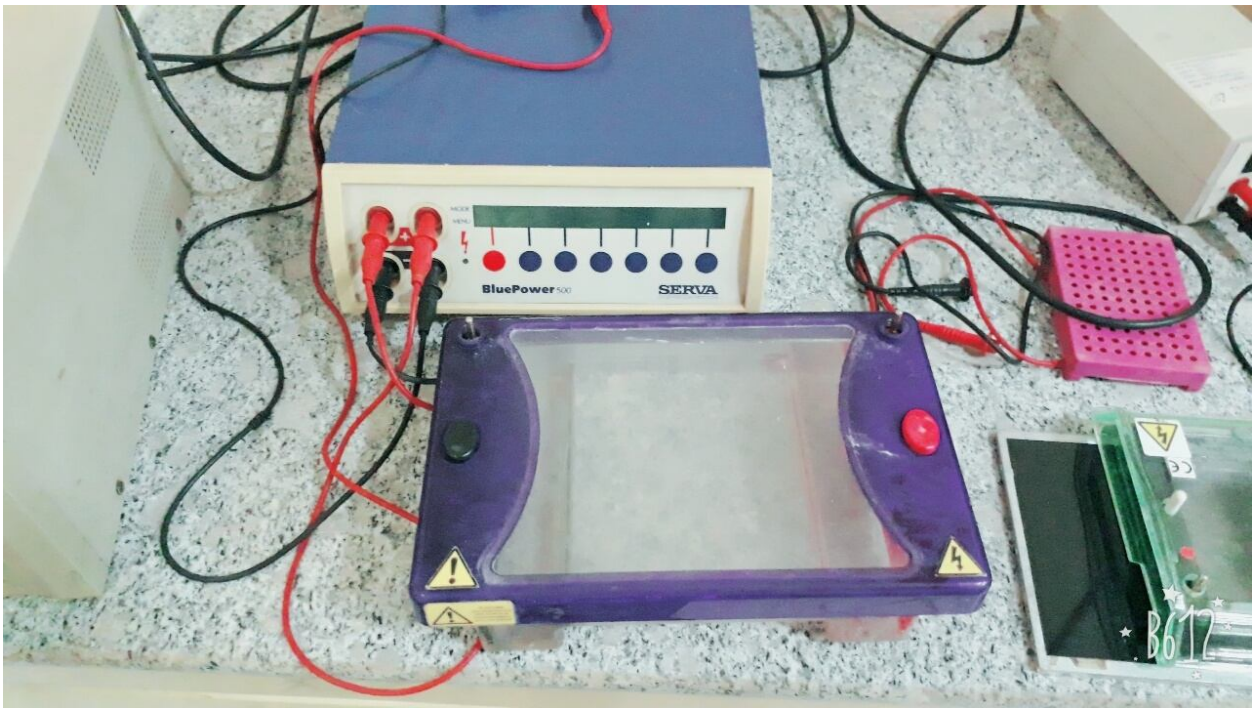

Figure (3) gel electrophoresis and power supply device

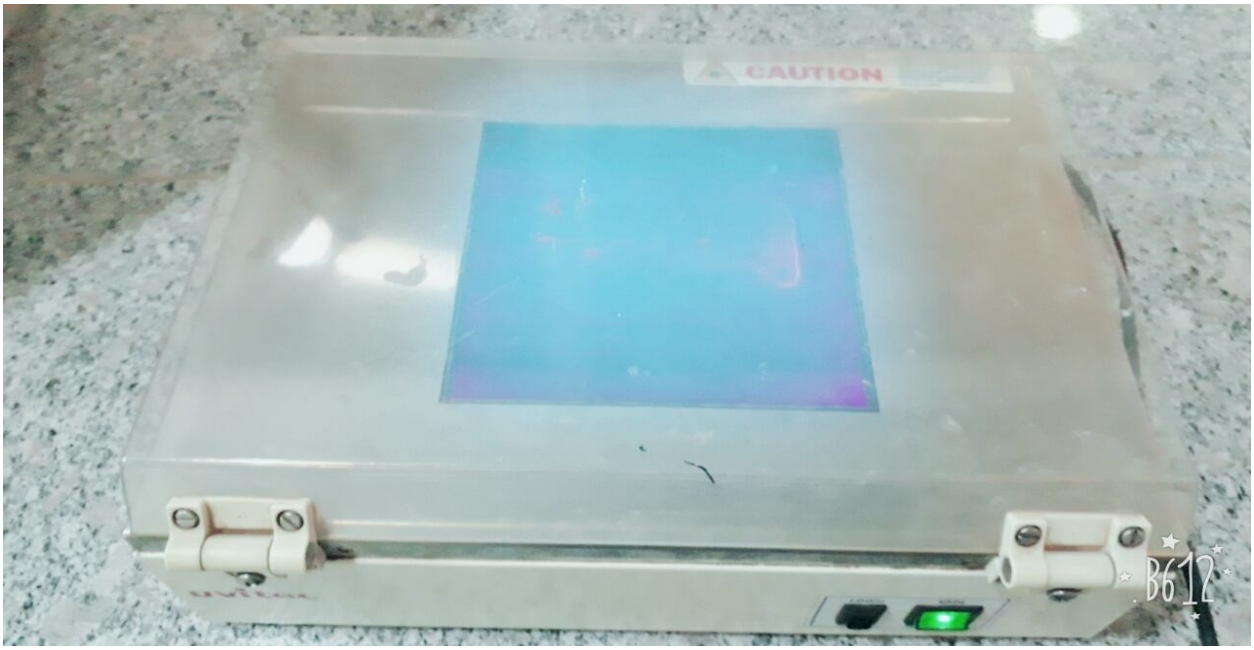

Figure (4) UV Light transilluminater device