Sudan University of Science and Technology

College of Graduate studies

Molecular Detection and Characterization of Diarrheagenic *Escherichia coli* from Clinical isolates in Khartoum State

الكشف الجزئي لبكتريا الاشركية القولونية المسببة للإ سهال المعزولة من العينات السريرية في ولاية الخرطوم

A dissertation submitted in partial fulfillment for the requirement of M. Sc degree in Medical laboratory science (Microbiology)

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August, 2017

الآيـــــــــــة

قال تعالى:

((وَاللَّهُ جَعَلَ لَكُمْ مِنْ بُيُوتِكُمْ سَكَنًا وَجَعَلَ لَكُمْ مِنْ جُلُودِ الْأَنْعَامِ بُيُوتًا تَسْتَخِفُّونَهَا يَوْمَ ظَعْنِكُمْ وَيَوْمَ إِقَامَتِكُمْ ۙ وَمِنْ أَصْوَافِهَا وَأَوْبَارِهَا وَأَشْعَارِهَا أَثَاثًا وَمَتَاعًا إِلَىٰ حِينٍ))

صدق الله العظيم

سورة النحل الآيــة 80

Dedication

To the candle of beauty and forgiveness

My family

To the supervisor how start with me step by step

Dr Hisham NourAldayem Altayeb Mohammed

To the meaning of honest

My friends

To everyone whom deserves this dedication

I dedicate this work

Acknowledgement

Firstly all thanks to Allah

I would thank my family for their patience

Special regard, respect for my supervisor Dr HishamNour-AldayemAltayeb Mohammed

For helping and skill full guidness

Also I would like to thank the staff of department of Microbiology in SUST for their love presented in this work

I would like to express my appreciations to all my friends

Thank so much to all of you

Abstract

Diarrheagenic *Escherichia coli* is one of the most important etiologic agents of several infections, and represent a major public health problem in developing countries like Sudan. The present study was undertaken to detect the virulence factors of diarrheagenic *E. Coli* strains using multiplex PCR in isolated samples of urine, high vaginal swab and wound swab. Fifty clinical isolate were collected from Omdurman military hospital and police hospital in Khartoum State; they were from 21(42%) males and 29(58%) females. Then they cultured on MacConkey agar and EMB media. Then biochemical tests were done, including indole test, citrate, Kilgelar iron agar and urease test. The sensitivity test was performed using Disc diffusion method; the following antibiotic discs were used (Ceftriaxone, Meropenem, Imipenem, Amikacin, Ceftazidime, Ciprofloxacin Gentamicin and Framycetin). Most of the isolated organisms were resistant to these antibiotics. DNA was extracted by boiling method. Multiplex PCR techniques was used to amplify four genes of diarrheagenic*E.coli*, the frequency of Enteroaggregative *E.coli* (*aggRgene*) was found in 8 (16) % in urine isolate , and negative in other isolates, Enterohermorrhagic *E.coli* (*stx*gene)was2(4%) in urine isolates and negative in other isolates,Enteroinvasive *E.coli* (*ipaH* gene) was (8) 16 % in urine isolate, and 2 (4%) in wound swab and 1(2%) in High vaginal swab. Enteropathogenic *E.coli*(*eae* gene) was negative in all isolates. From this study we concluded that, the presence of diarrheagenic *E.coli*in other samples than stools may indicate the source of infection and the predominant strain is Enteroinvasive *E.coli* .

ملخص الاطروحه

أجريت هذة الدراسة للكشف عن عوامل الضراوة لبكتريا الاشريكيه القولونية المسببه للاسهال, تم جمع عدد 50 عينة معزوله من البول، ومسحات عمق الرحم ومسحات الجروح من مستشفى السلاح الطبي ومستشفى الشرطه في ولاية الخرطوم. وكان عدد الذكور 21 (42٪) والإناث 29 (58)٪ , وتم التعرف علي البكتريا عن طريق زراعتها في وسط MacConkey و EMB وتم اجراء التفاعلات الكميائيه الحيويه التي تشمل indole و citrateو Urease و Kilgelar iron agar واجريت ايضاً إختبارات الحساسيه بواسطه إستخدام المضادات الحيويه التالية: السفترايكزون, الميروبينيم, الاميبينيم, السبروفلوكساسين, السيفتازيديم, الاميكاسين والفرامايستين). وكانت معظم العزلات مقاومه لمعظم المضادات الحيويه المستخدمه وبعد ذالك تم إستخلاص الحامض النووي للبكتريا باستخدام طريقه الغليان ثم بعد ذالك تم الكشف عن وجود 4 انواع مختلفه من جينات الضراوه للاشريكيه القولونيه باستخدام تفاعل البلمرة المتعدد , وكانت النتائج إيجابيه لجينaggR في 8 من البكتريا المعزولة من عينات البول بنسبه 16٪ ونتائجه سلبية في البكتريا المعزوله من العينات الاخرى ، جينSTXاعطى نتائج ايجابيه لعينتين فقط بنسبة 4٪ في البكتريا المعزوله من البول ونتايج سلبية للعينات المعزوله من عينات أخرى. جينIpaHكان ايجابي في عدد8(16٪)من البكتريا المزوله من البول2(4٪) للبكتريا المعزوله من مسحات الجروح و1(2٪) للبكتريا المعزولة من مسحات عمق الرحم وجين eae كان سالب في جميع العينات.ومن هذه الدراسه خلصنا الي ان البكتريا المسببه الي الاسهال يمكن عزلها من عينات اخري غير عينة الفسحه واكثر نوع تم عزله هو جين. EIEC.

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Abbreviations

EAEC	Enteroaggregative E.coli
EHEC	Enterohemorrhagic E.coli
ETEC	Enterotoxigenic E.coli
DAEC	Diffusely adherent E.coli
UPEC	Uropathogenic E.coli
EIEC	Enteroinvasive E.coli
EPEC	Enteropathogenic E.coli
ExPEC	Extraintestinal pathogenic E.coli
VTEC	Verotoxigenic E.coli
NMEC	Neonatal Meningitis-causing E.coli
SEPEC	Sepsis-causing E.coli
IPEC	Intestinal pathogenic E.coli
IPM	Imipenem
CRO	Ceftriaxone
MEM	Meropnem
AK	Amikacin
CIP	Ciprofloxacin
CAZ	Ceftazidime
GN	Gentamicin
F	Framycetin

CHAPTER ONE
INTRODUCTION

Chapter one

1.1 Introduction

E. coli is one of the most extensively studied Gram-negative bacteria in microbiology. This species has been associated with intestinal and extraintestinal infections in humans and many animals, (Toval *et al*., 2014).The major groups of intestinal pathogenic *E. coli* (IPEC) have been recognized are Enteropathogenic *E. coli* (EPEC), Shiga Toxin-producing,*E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). In addition, three types of Extraintestinal Pathogenic *E.coli* (ExPEC), including Neonatal Meningitis-Causing *E.coli* (NMEC), Sepsis-Causing *E.coli* (SEPEC), and Uropathogenic *E. coli* (UPEC), have been associated with meningitis in newborns, systemic infections, and urinary tract infections (UTIs), UPEC causes around 90% of community-acquired UTIs and up to 50% of nosocomial UTIs; It has been estimated that catheter-associated UTIs represent one of the most common causes of nosocomial infection and that treatment costs are as high as $400 million annually in the United States. The molecular epidemiological studies assessing the population structure of UPEC isolates from nosocomial UTIs or from hospital inpatients and outpatients have not been performed to date. Most UTI cases have been reported for women, children, elderly people, and immunocompromised patients. Combination of various risk factors represents the most plausible cause of such a phenomenon, including physiological and anatomical changes, an active sex life, age, and the close proximity of the urethra, vagina, and rectum in women. It is broadly accepted that the fecal flora of the host is the primary source of UPEC isolates, despite the fact that UPEC and ExPEC in general belong to the normal intestinal flora of many healthy individuals,

where they coexist with commensal *E. coli*. They are often distinguished based on their virulence gene contents and their allocation to certain phylogenetic lineages, however,in hospital settings, immune-compromised patients are, having indwelling urinary catheters, and are exposed to a plethora of antimicrobial compounds that might promote UTIs caused by various *E. coli* strains with an unusual virulence gene (Toval *et al*., 2014). Conventional diagnostic methods, such as culture, biochemical tests, and enzyme-linked immunosorbent assay (ELISA),are methodsof diagnosis and then developed to use molecular detection of a multiplex PCR assays to screen for several genes of the major diarrhea-causing bacteria(Liu *etal*.,2011).

1.2. Rationale:

Diarrheal diseases remain one of the leading causes of preventable death in developing countries, especially among children under 5 years of age. In 2008 alone, around nine million children under 5 years, died and around 40% of those deaths were due to pneumonia and diarrhea (You *et al.*, 2010). Globally, diarrhea is the second largest cause of death in children under 5 years of age, causing one in every five deaths. Unfortunately, diarrhea kills more children than AIDS, malaria and measles combined Diarrhea is common in the developing countries, especially in areas with poor hygiene and sanitation and with limited access to safe water. Other conditions, such as malnutrition, may further increase the risk of contracting diarrhea in developing countries. These factors may lead to a significant disease burden and negative economic effects, resulting from medical costs, loss of work, lower quality of life and high mortality (Saeed *et al* ., 2015).

Diarrheogenic *E.coli* become a major health problem in the world while , in the Sudan the prevalence represent 48 % from cases suffering from diarrhea, In Sudan infant mortality is 102 per 1000 live births and neonatal mortality is 51 per 1000 live birth (Eltayeb *et al*., 2014).

Hence there is major complication including bacteraemia, sepsis and meningitis capable of causing infections in the elderly and the immunosuppressed, including people with diabetes, HIV and chronic heart disease (Ahmed *et al.*, 2014).Hemolytic–uremic syndrome began in northern Germany cases have subsequently been reported in 15 other countries (Rasko*et al.*, 2011).

Also induce inflammatory responses and cytoskeletal dynamics(Oliveira *etal*., 2011).

1.3. Research Questions

1- Does diarrheagenic *E.coli* present in other specimens than stool

2- What is the frequency of diarrheagenic *E.coli* in each sample.

3- What are the most common genes responsible for disease causing by diarrheagenic *E.coli.*

4- Is there any association between the presence of diarrheagenic *E.coli* gene and type of infection, antibiotics resistance and gender.

1.4. Objectives

1.4 .1 General Objective

Molecular detection of diarrheagenic *Escherichia coli* isolated from clinical samples (Urine, High vaginal swabs and Wound swabs) from different hospital of Khartoum State.

1.4.2 Specific Objectives

1- To screen for the presence of diarrheagenic *E.coli* genes in clinical sample in Khartoum State.

2- To determine the most common genes of diarrheagenic *E.coli* associated with infection.

3- To associate between the presence of diarrheagenic *E.coli* genes and antibiotic resistance in Khartoum State.

4- To associate between diarrheagenic *E.coli* genes and type of infection.

CHAPTER TWO

LITRETURE REVIEW

Chapter two

2.1. Definition

Diarrheagenic *E. coli is an E.coli* produces toxins or possesses certain virulence traits. (EHEC) produce toxins, heat-labile (LT) and/or heat-stable toxin (ST) and shiga-like toxins I and II (SLT I/II), respectively. (EIEC) typically invade and destroy the bowel mucosa. (EPEC) damage the bowel mucosa with characteristic attaching and effacing lesions mediated by a protein encoded by a gene called the attaching and effacing locus .

Verotoxigenic *Escherichia coli* (VTEC), verotoxin production has been identified in more than 200 serogroups and the incidence of this syndrome in children is increasing hemolytic uremic syndrome is the most common cause of acute renal failure in children worldwide are named for their ability to produce toxins with a cytopathic effect on Vero cells. Diarrheagenic *E. coli* organisms have an important role as a cause of enteric diseases. However, these pathogens are probably under estimated due to inappropriate diagnostic methods in clinical practice (Arnada *et al*., 2007).

2.2 Etiology

E. coli is the most abundant facultative anaerobe, most are commensals in the human intestinal micro flora, but certain strains have virulence properties that may account for life threatening infections. The pathogenicity of a particular *E.coli* strain is primarily determined by specific virulence factors which include adhesion, invasion, haemolysin, toxin, effacement factors, cytotoxic necrotic factors and capsules and these have been implicated in human and animal disease worldwide with the pathogenic strains being categorized into intestinal pathogenic *E.coli* (InPEC) and extra – intestinal pathogenic *E.coli* (ExPC) on basis of their virulence factors and clinical symptoms (Titilawo *etal*.,2015).

A-**Enterotoxigenic *E.coli* (ETEC)**

ETEC is one of the most studied pathotypes of DEC. Most studies have demonstrated the association of ETEC with diarrhea among infants less than five years of age (Aguero *et al*.,1985; Shaheen *et al*., 2009). Most of the illness, both in terms of number of cases and severity of symptoms, occur in infants after weaning. Moreover, epidemiological studies have implicated contaminated food and water as the most common vehicles of ETEC infection. A high infectious dose (ranging $10^6$to 10^{10} CFU) of ETEC has been demonstrated in human (Levine *et al.*, 1979;Nataro and Karper 1998), and a large number of serogroups of ETEC have been associated with diarrhea (Stenutz *et al*, 2006).

B-Enteropathogenic *E.coli* (EPEC)

EPEC was the first pathotype of DEC described, and associated to the infant diarrheal diseases worldwide (Nataro and Karper,1998). The characteristic intestinal histopathology .attaching and effacing (A/E) lesions - of EPEC are associated to striking cytoskeletal changes in the epithelial cell. This ability to induce-attaching and effacing lesions is encoded by genes for the adherence factor intimin **(***eae***),** a type II secretion system (TTSS) that includes the *esc* genes and the translocated intimin receptor (Tir) (Nataro and Karper,1998). These genes are located on a 35-Kb (kilobase) pathogenicity island (PAI), called the locus of enterocyte effacement (LEE), which is present in all EPEC and EHEC (McDaniel *et al.*, 1995).The A/E mechanism induce microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at the site of bacterial attachment (McDaniel *et al.*, 1995; Nataro and Karper, 1998).

C-Enteroaggregative *E. coli* (EAEC)

EAEC was first described in 1985, recognized by its distinctive adherence to HEp-2 cells in an aggregative, stacked brick-like. Pattern (Nataro *et al*., 1998; Pereira *et al*., 2008). This adherence pattern, distinguishable from the adherence patterns manifested by EPEC and DAEC, was first significantly associated with diarrhea among Chilean children in 1987 (Nataro *et al*., 1987). EAEC is defined as an *E. coli* pathotype that does not secrete heat labile or heat-stable enterotoxins but adheres to HEp-2 cells and other epithelial cells in an aggregative adherence (A/A) pattern, the genes for which are encoded on plasmids (Nataro *et al*., 1998).Many epidemiological studies have used the A/A pattern and the plasmid-encoded probe .CVD432. or simply the A/A probe to identify EAEC (Baudry *et al*., 1990). Moreover, atranscription activator known as .*AggR*., the gene which regulates the A/A genes has been described as the major EAEC virulence regulator (Nataro, 2005). Recently, some epidemiological studies have suggested that CVD432-positive strains, which are predicted to carry the *AggR* regulators, are the true EAEC pathogens termed ,typical EAEC, (Harrington *et al*., 2006). However, A/A probe negative isolates share virulence factors with A/A probe positive isolates, a finding which indicates that additional factors are involved in the A/Aphenotype in these EAEC strains (Bouzari *et al*., 2001).

D-Enterohemorrhagic *E.coli* (EHEC)

EHEC is an etiological agent of diarrhea with life-threatening complications. EHEC belong to a group of *E. coli* called VTEC (verotoxigenic *E. coli*. or Verocytotoxin-producing *E.coli*.) or STEC (Shiga toxin-producing *E. coli*), formerly SLTEC (Shiga-like toxin producing *E. coli*.). EHEC colonize the intestinal mucosa inducing a characteristic attaching and effacing (A/E) lesion, which is also present in EPEC and EAEC. The classic intestinal histopathology characteristic of EHEC infection includes hemorrhage and

edema in the lamina propria, which results in bloody diarrhea, hemorrhagic colitis, necrosis and intestinal perforation. The major virulence factor, and a defining characteristic of EHEC, is the Shiga toxin (Stx), a potent cytotoxin that leads to cell death and will aggravate the symptoms in patients infected (Nataro and Karper,1998).

E-Enteroinvasive *E.coli* (EIEC)

Like most enteropathogens, these bacteria may also be important pathogens in developing countries where sanitation and hygiene levels have deteriorated. EIEC strains are biochemically, genetically, and pathogenically closely related to *Shigella* spp., but produce less severe diarrheal disease, compared to the dysentery caused by *Shigella dysenteriae*type1 (Nataro and Karper,1998). The precise pathogenetic scheme of EIEC has yet to be elucidated. However, pathogenesis studies of EIEC suggest that its pathogenic features are virtually those of *Shigella* spp. (Sansonetti, 1992). EIEC invades the colonic epithelial cell, there by inducing an inflammation and mucosal ulceration, leading to the release of blood and mucus in the stool (Hart *et al*, 1993), similar to bacillary dysentery.

F- Diffusely adherent *E. coli* (DAEC)

DAEC is a category of DEC that produces the diffuse adherence on Hep-2 cells (Nataro *et al*. 1998) similar to those EAEC strains. However, little is know about the pathogenesis of DAEC, but cells with a surface with fimbriae that mediate diffuse adherence have been cloned and characterized (Kaper *etal*., 2004). Few epidemiological and clinical studies have been carried out to adequately describe the epidemiology and clinical aspect of diarrhea caused by DAEC.It was suggested that DAEC may cause disease in immunologically naïveor malnourished infants. Furthermore, DAEC has been associated to diarrhea in infants older than 1 year of age (Scaletsky *et al*., 2002).

These diarrheagenic.*coli* (DEC) strains usually play important roles as the causes of endemic and epidemic human diseases, such as severe diarrhea, food poisoning, and similar outbreaks worldwide (Yang*et al*., 2007). Also cause a wide spectrum of intestinal and extraintestinal infections, such as diarrhea, urinary tract infection, meningitis, and septicemia (Sidhu*et al*., 2013). The spectrum of pathogens responsible for such infections varies with age and geographical location (Hardegen*et al*., 2010).

2.3 Pathogenesis

The major distinguishing factor between pathogenic and non-pathogenic strains of *E. coli* strains is the occurrence of virulence genes, which code for the various known strategies for pathogenecity (Bisi *et al*., 2011).These virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics (Rono *et al*., 2014).

Enteroinvasive *E.coli*, Enterohemorrhagic *E.coli*,Enteropathogenic*E.coli* , and Enteroaggregative *E.coli* this group, called diffusely adhering *E. coli* (DAEC), has been identified as a putative new pathogenic group, Most of the pathogenic *E. coli* strains bind to epithelial cells. The adhesion is mediated by bacterial proteins designated as adhesions, which can be differentiated on the basis of their binding receptor specificity. It is possible to reproduce invitro the ability of the bacteria to adhere by using cultured epithelial cells. Three distinct patterns of adherence have been described: localized, aggregative, and diffuse. Localized adherence is characteristic of the (EPEC) and (EHEC) strains, the aggregate pattern of adherence is described for the (EAEC) strains, and the (ETEC) and (DAEC) strains adhere uniformly on the cultured cells with a diffuse adherence pattern. Studies on the adherence properties of *E. coli* it has yielded conflicting results. These results could be attributable to the different origins of the strains tested (stool samples, rectal biopsies),

technical differences in cell adhesion procedures, or differences caused by the epithelial cell type used. *E. coli* is distinct in their adherence pattern with a so-called stack-brick adherence to Hep-2 cells. In addition, (EAEC) may harbor additional virulence factors, including fimbriae I and II (AAFI and AAFII), an ST-like enterotoxin (EAST), and a plasmid-encoded heat-labile toxin (PET) that are involved in aggregation and pathogenesis of the associated diarrhea. (Presterl *etal.*, 2003).ETEC strains cause cholera-like watery diarrhea through the elaboration and action of LT(heat-labile) and/or ST (heat-stable) enterotoxins. The ability of ETEC strains to produce diarrheal illness by either or both of these enterotoxins is what defines an ETEC. However, to cause diarrhea, by LT and/or ST, the ETEC must adhere and colonize the intestinal mucosa. This is achieved by attaching with one or more colonization factor antigens (CFAs), which are antigenically diverse and usually are encoded by plasmids (Nataro and Karper, 1998).

2.4 Epidemiology

The diarrheagenic *E. coli* (DEC) strains usually play important roles as the causes of endemic and epidemic human diseases, such as severe diarrhea, food poisoning, and similar outbreaks worldwide (Yang *et al*., 2007).The Diarrheal diseases are major causes of morbidity, with attack rates ranging from 2 to 12 or more episodes per person per year, especially in developing countries (Nguyen *et al*., 2005). Besides the classic pathogens *Shigella*, *Salmonella*, *Yersinia*, *Vibrio*, and *Campylobacter spp*, at least five different categories of *Escherichia coli* may cause diarrhea worldwide,(ETEC), (EHEC), (EPEC), (EIEC), and (EAEC). The associated clinical pictures comprise childhood and traveler's diarrhea (ETEC), bloody diarrhea and hemolytic uremic syndrome (EHEC), infantile diarrhea (EPEC), and bacillary dysentery-like diarrhea (EIEC).EAEC have been associated with acute and persistent diarrhea in children and adults in industrial and developing

countries in Europe, America, Asia and Africa (Presterl *et al*., 2003). The most recently recognized categories of diarrheagenic *E.coli*, adhere to tissue culture cells in an aggregative adherence pattern and are associated with persistent, watery diarrhea in young children in India, Mexico, and Brazil with occasional bloody diarrhea cases (EAggEC) may also associate with childhood diarrhea in England. That affecting different sex and all age groups across the life span, but women is more susceptible than men, due to shorter urethra, absence of prostatic secretion, pregnancy and easy contamination of the urinary tract with fecal flora (Panvadiwala *et al*., 2016).The annual incidence of (EAEC) infections ranges from 0.1 to 12 cases per 100 000 population. (Ahmed *et al*., 2004). In addition, diarrheal illnesses account for an estimated 12,600 deaths each day in children under 5 years of age in Asia, Africa, and Latin America (Nguyen *et al* ., 2005).In British Columbia (BC), Canada, the rate of STEC infections has remained above the Canadian average since 2004, ranging between 2.4 and 4.3 cases/ 100,000 individuals .(Chandran and Mazumder,2015). In Mexico it is the second cause of morbidity and the fifth cause of mortality among children under five years of age. In 2010, more than 5.5 million diarrheal cases were reported for a national rate of 5,264.24 per 100,000 individuals (Patzi-vargas *et al.*, 2015).

2.5Risk group of Diarrheagenic *E.coli*

Diarrheagenic *E. coli* strains are responsible for 30%–40% of cases of acute diarrhea in children (Ochoa *et al*., 2009). Recent data suggest that these strains are more common in the United States in children less than 5 years of age with acute diarrhea (Guion *et al* ., 2008) .

2.6 Methods of diagnosis

2.6.1 Culture

2.6.1.1 MacConkey Agar

E.coli cultivated on MacConkey agar. After overnight incubation at 37°c, lactose fermenting colonies (LFC) with the typical appearance of *E. coli* were selected for further analysis. Isolates that can be identified by biochemical assays (Bisi-Johnson *et al*., 2011).

2.6.1.2 Eosin Methylene Blue Agar

EMB agar medium contained lactose and the dyes eosin and methylene blue that permitted differentiation between enteric lactose fermenters and non fermenters as well as identification of the colon bacillus *E.coli*. Colonies were small, dark centered with greenish metallic sheen caused by the large quantities of acid that was produced and precipitated out the dyes onto the growth surface on EMB (Panvadiwala *et al* .,2016) .

2.6.2 Biochemical reaction

E. coli can be identified by several biochemical reactions, including sugar utilization; lysine carboxylation, indole production, and the motility test (Yang et *al*., 2007).

2.6.3 Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was determined using the agar serial dilution method according to CLSI standards (Cao *et al*., 2011). *E. coli* ATCC 25922 was run in parallel for quality control, and the results were analyzed and interpreted according to CLSI guidelines. Due to the absence of a CLSI breakpoint for the interpretation of moxifloxacin results, the interpretation was made using the current European Committee on Antimicrobial Susceptibility Testing (Cao *et al*., 2011)

2.6.4 Extraction of DNA

Each sample was inoculated onto sorbitol MacConkey agar and incubated for 18-24 hours at 35-37°C. A loopful of bacterial colony from each sample was suspended in 200µl sterile deionized water in 1.5ml eppendorf tubes and adjusted to 0.5 McFarland. This was mixed thoroughly by vortexing, boiled at 95-100oC for 10 minutes and then cooled to room temperature (RT). The suspension was centrifuged at 10,000 rpm for 1min in RT and the supernatant DNA extract pipetted into 1.5ml microfuge tube and stored at -20. To confirm (EHEC, ETEC, EIEC, EAEC and EPEC) strains, multiplex PCR assays (Rono *et al*., 2014).

2.6 .5 PCR amplification

PCR for rapid detection and identification of the specific intimin alleles in *E. coli* strains, to accomplish this, they designed oligonucleotide primers for multiplex PCR based on the multiple sequence alignment (Reid *et al*., 1999). The multiplex PCR assay was carried out with a 50 ml of reaction mixture containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 2mM of each deoxynucleoside triphosphate, 1.5U of AccuPrimeTaq DNA polymerase (Invitrogen), 2 mL of the DNA template and the PCR primers. The optimal concentration of each primer pair in the reaction mixture was determined empirically. Each primer pair concentration was varied independently until the PCR products exhibited equal intensities on 2% agarose gels when a DNA mixture of five prototype E. coli strains was used as the PCR template the concentration for each primer pair in the final reaction is given in Table 1. The PCR mixtures were then subjected to the following cycling conditions: 50 1C (2 min, 1 cycle), 95 1C (5 min, 1 cycle), 40 cycles of 95 1C (1 min), 50 1C (1 min), 72 1C (1 min) and 72 1C (7 min, 1 cycle) in a thermal cycler (model system 2400; Perkin-Elmer Corporation, Norwalk, Conn.). PCR products (10 ml) were visualized after electrophoresis in 2% agarose gels in

Trisborate- EDTA buffer and ethidium bromide staining, and the amplicons were identified based only on the size of the amplified product. In all further experiments, the DNA mixture of the five diarrheagenic reference strains from pure cultures served as the positive control, while E. coli K12 DH5a was the negative control (Arnada *et al*., 2007).

2.7 Treatment

Diarrheagenic *E. coli* strains are not routinely sought as stool pathogens in clinical laboratories. Some of these pathogens respond to antimicrobial agents, while for others (e.g., Shiga toxin-producing *E. coli* STEC),

Antibiotics should be avoided. Because the time frame in which treatment choices must be made is short. (Guion *et al*., 2008). The use of antibiotics or ant motility/ant diarrheal and antimicrobial agents in early stages of diarrhea has been shown to increase the risk of hemolytic uremic syndrome because the gut is exposed to a greater number of toxins for a longer period as intestinal motility slows. The pathogen is relatively tolerant to acid and can survive in fermented foods and fresh vegetable produce. (Navidinia *et al*., 2012).

2.8 Resistant to Antibiotic

E.coli resist the effects of antibiotics in four ways. First, by producing antibiotic inactivating (or -modifying) enzymes, such as beta-lactamases, which deactivate the drug by cleaving to the core beta-lactamase ring structure. Extended-spectrum beta-lactamases (ESBLs) mediate resistance to penicillin and third-generation cephalosporins. *Escherichia coli* also resist the effects of antibiotics by restricting the concentration of antibiotics within the cell by reducing membrane permeability and resisting entry of a wide range of antibiotics. A third mechanism of resistance is alteration of the antibiotic target site so the antibiotic is unable to bind properly. This type of mechanism is used by some strains of *E. coli* to resist quinolones and macrolides. The

fourth type of resistance mechanism is to eliminate the antibiotic target site entirely, either through over producing the target enzyme or producing an alternative target enzyme. Some resistance mechanisms confer resistance to only one class of antibiotics while other mechanisms confer resistance to many classes of antibiotics. One strain of bacteria may possess several different types of resistance mechanisms making it multi-drug resistant (Coleman, 2008).

CHAPTER THREE

MATRIALS AND METHODS

Chapter three

3. Materials and methods

3.1. Type of study

This is Cross sectional hospital based study

3.2. Study area

Data o f this study was collected from Omdurman military hospital and police hospital in Khartoum State

3.3. Study population

The isolates were collected from Omdurman military hospital and police hospital from patients suffering from urinary tract infection, genitourinary tract and wound infection.

3.4 Study duration

This study was carried out during 4 months between June and August, 2016

3.5 Data collection

The data was collected from Khartoum hospitals laboratory data records

3.6 Ethical consideration

Permission to carry out this study was obtained from College of Graduate Studies, Sudan University of Science and Technology. All patients were informed about the purpose of the study before collection the samples.

3.7. Sample size

Fifty isolates were collected from hospitalized patients.

3.8 Collection of isolates

The isolates were collected from hospitals in Khartoum State.

3.9 Identification of isolates

3.9.1 Gram‘s stain

From overnight culture, smear was done by adding small portions from the colony by sterile loop to drop of normal saline on clean, dry slide then spread evenly and left to dry and then fixation was done by gentle heat(Appendix II).

3.9.2. Biochemical tests

The biochemical tests were done including the following

3.9.2.1. Citrate utilization test

By using of sterile loop under aseptic condition the organism under test was inoculated in Simmon’s citrate and incubated at 37◦C for 96 hours.(Appendix I).

3.9.2.2. Urease test

By using of sterile loop under aseptic condition the organism under test was inoculated in urea media vertically. The tubes were incubated at 37◦C for overnight (Appendix I).

3.9.2.3. Indole test

By using of sterile loop under aseptic condition the organism under test was inoculated in Trypton water, the tubes were incubated at 37◦C for overnight after incubation period. Drops of Kovac‘s reagent was added (Appendix I).

3.9.2.4. Kiligler Iron Agar (KIA)

By using of sterile loop under aseptic condition the organism under test was inoculated inthe butt of KIA tube and making zigzage on the slope of the media 37◦C and incubate for overnight. (Appendix I).

3.9.3 EMB media

By aid of sterile standard bacteriological loop, the agar was inoculated under aseptic condition near the flame. The inoculated media were incubated aerobically at 37◦C for overnight (12-24) hours. The preparation of media in the appendix. After the incubation period, the plates were examined for the presence of green metallic sheen that was significant for *E.coli*.

3.10. Susceptibility testing

Kirby Bauer disc diffusion method was use to perform susceptibility test as follows:

Standard control strains were prepared in sterile normal saline in a test tube. The density of suspensions was adjusted to McFarland turbidity standard. Then under aseptic condition near the flame the plates of Muller Hinton agar (appendix) were seeded by suspension by means of a cotton wall swab. The seeded plates were incubated at room temperature for 5 minutes. Then the following antibiotic discs were applied Ceftriaxone 30mcg, Meropenem 10mcg, Amikacin 30mcg, Ciprofloxacin 5mcg, Ceftazidime 30mcg, Gentamicin 10mcg and Framycin 100mcg. The plates were then incubated aerobically at 35◦C for 16-24 hours and at the end of incubation period the zones of inhibition were measured in mm, and aligned to manufacture chart (Bauer *et al.*, 1966).

3.11 Extraction of DNA

A pure colony of *E.coli* was cultured in Nutrient Agar media and incubated aerobically at 37˚C for overnight and after that heavy inoculums of bacteria was taken and put in an eppendorf tube that contain 1ml of normal saline and using of vortex was mixed gently, then was centrifuged the mixture for 5 minutes at 3000r/m, the supernatant was discharged, the pellet was

resuspended in 200µLof DW and boiled for 95◦C for 15 minutes, then centrifuged at 13,000/min for 3minutes, the supernatant that contain DNA was transferred to another tube (Sherfi, 1998).

3.12 Measurement of DNA

Concentration of extracted DNA was read using the gel electrophoresis (Appendix III).

3.13 Polymerase Chain Reaction (PCR)

3.13.1. Primers

PCR amplification was performed using published primer pairs (Ndlovu *et al*., 2015) which are shown in table 1.

Table 1. Primers sequences used for detection of diarrheagenic *E.coli* genes

Use of different sequences length according to specific primer the length is 254,518,619 and 881bp

Gene detected	Primer name		Primer sequence	Product size(bp)	Annealing Temp(◦C)	References
aggR	**aggRks 1** **aggRks 2**	F R	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	254	55	Ndlovu *et al*., 2015
Stx	**VTcom-u** **VTcom-d**	F R	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	518	55	Ndlovu *et al*., 2015
ipaH	***IpaH* 1** ***IpaH*2**	F R	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	619	55	Ndlovu *et al*., 2015
Eae	**SK1** **SK2**	F R	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881	55	Ndlovu *et al*., 2015

F= Forward

R=Reverse

3.13.2 Preparation of primers

For 100pmol/µ from each primer was dissolved in DW as follows:

AggRks in 220µl DW, VTcom (stx) in 220 µl DW, IpaH in 250µl DW , SK in 250 DW . And for 10 pmol/ µL, 10 µl of each primer was dissolved in 90 µl of DW.

3.13.3Preparation of 10 X TE buffer

Amount 108 grams of Tris base were added to 55 g of boric acid and 40ml of 0.5 EDTA, then into liter deionized water pH 8.0.

3.13.4Preparation of 1X TE buffer

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

3.13.5 Preparation of ethidium bromide

Five milligrams of ethidium bromide was dissolved in 500 µl DW and kept in brown bottle.

3.13.6 Preparation of Agarose Gel

Amount of 2 g of agarose powder was dissolved in 100ml 1X TE buffer and heated in microwave for 1 minute. Then the mixture was cooled to 55 ˚C in water bath. Then 2.5 µl of (20mg/ml) ethidium bromide were added, mixed well and poured into a casting tray that was taped up appropriately and was equipped with a 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 slides was removed.

3.13.6Master mix

Maxime PCR premix (Intron biotechnology Korea) which contain (Tag polymerase 5U/µl ,dNTPs , reaction buffer (10x) and gel loading buffer) was premixed, ready to used solution containing all reagents required for PCR (except template DNA and primers) and additional compound needed for direct loading onto agarose gel and two tracking dyes (blue and yellow)that allow monitoring progress during the electrophoresis.

3.13.7Preparation of reaction mixture

The following reagents were used for each gene in following volumes (total reaction volume 25µl) in 0.5ml eppendorf tube;

1. 17.0 µl deionized sterile water
2. 5µl Maxime PCR premix (Intron biotechnology)
3. 0.5µl Forward primer (Intron, Korea).
4. 0.5µlReverse primer (Intron, Korea)
5. 2µl DNA(Template DNA)

3.13.8 .Protocol used for amplification of four genes (AggRKs, VTcom (stx),KS(eae), IpaH)

Multiplex PCR was used of a Thermocycler (Teche Tc-312, UK). The PCR mixture was subjected to initial denaturation step at 94˚Cfor 5 min , followed by 30 cycles of denaturation at 94˚C for 45 second, primer annealing at 50˚C for 45 second , followed by the step of elongation at 72˚C for 60 seconds, and the final elongation at 72˚C for 5 min (Arnada *et al*.,2007).

3.13.9 Visualization of PCR products

The gel tray was covered with10X TBE buffer up to surface, then 3µl of each PCR product was loaded into each well. Then to the first well of casting tray 3µl of DNA ladder (100bp) was injected for each run. The electrophoresis apparatus was connected to the power supply (primer, 125v, 500mA, UK).The electrophoresis was done at 100v for 10min, after that the gel was removed by gel holder and visualized by U.V transilluminater (Uvite-UK). The results were photographed using gel documentation system (Appendix III).

3.14Data analysis

Statistical analysis was performed using SPSS software; version 16.0 and the result were presented in aform of tables and figures. Chi square was used, p-value < .05 was considered significant.

CHAPTER FOUR

RESULTS

Chapter four

Results

4.1 Gender

Among the study population (50 patients) there were 29 (58%) females and 21(42%) males, figure 1.urine, high vaginal swab and wound swab were collected as in figure 2

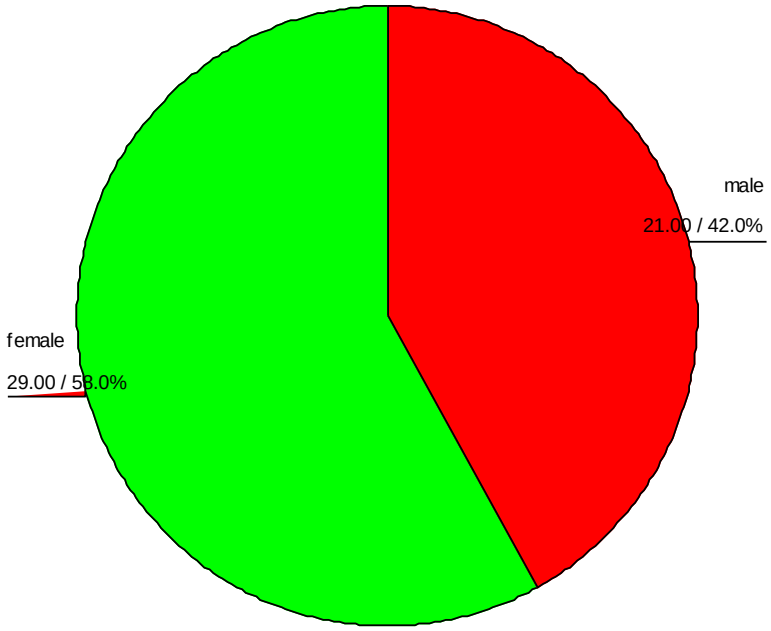

Figure 1. Distribution of enrolled patients according to gender

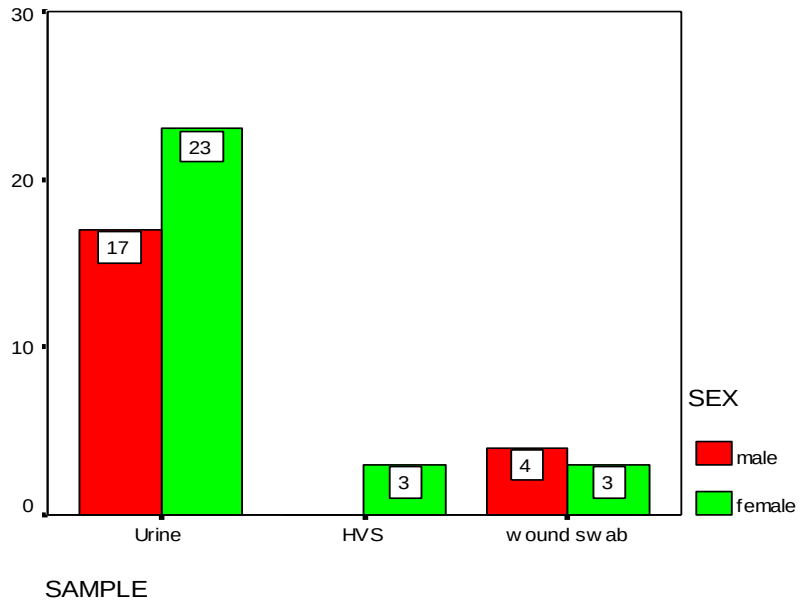

Figure 2. Distribution of sample according to gender

4.2. Gram´s stain

The Gram´s stain (Appendix) was done to 50 isolates and all the isolates gives Gram negative bacilli Appendix III.

Biochemical tests

Table 2. Biochemical tests were performed for all 50 isolates and the result show

Organism name	Indole test	Citrate test	Urease test	KIA			
				S	B	H2S	G
E.coli	+	-	-	Y	Y	-	+

+= positive G = Gas

S= Slope Y = Yellow

B = Butt

4.4. EMB Media

All isolates when cultured on EMB media and yield green metallic shine colonies seen in Appendix III

Sensitivity testing

The *E. coli* isolated was more sensitive to Imipenem (52%),Meropenem (50%) and Amikacin (46%) and less sensitive to Gentamicin (36%), Framycin (32%), Ciprofloxacin (28%), Ceftazidime (24) and Ceftrixone (16%), (Table 3). There was significant association between the presence of *AggR* gene and antibiotic sensitivity the P-value <0.05 (Table 4).

4.5 Susceptibility testing

Table 3. Show the frequencies of antibiotic according to specimens

Sample	**Antimicrobial susceptibility test No (%)**															
	IPM		**CN**		**AK**		**F**		**CRO**		**CIP**		**MEM**		**CAZ**	
Urine	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
	26 (52)	14 (28)	18 (36)	22 (44)	23 (46)	17 (34)	16 (32)	24 (48)	8 (16)	32 (64)	14 (28)	26 (52)	25 (50)	15 (30)	12 (24)	28 (56)
HVS	0 (0)	3 (6)	1 (2)	2 (4)	2 (4)	1 (2)	3 (6)	0 (0)	0 (0)	3 (6)	1 (2)	2 (4)	1 (2)	2 (4)	1 (2)	3 (6)
Wound swab	5 (10)	2 (4)	5 (10)	2 (4)	4 (8)	3 (6)	3 (6)	4 (8)	3 (6)	2 (4)	2 (4)	5 (10)	3 (6)	4 (8)	1 (2)	7 (14)
Total	31(62)	19(38)	24(44)	26(52)	29(58)	21(42)	22(44)	28(56)	11(22)	38(74)	17(34)	33(66)	29(58)	21(38)	14(28)	38(74)

Diarrheagenic *E. coli* genes isolated from urine sample more sensitive to Imipenem and Meropnem and strong resistant to Ceftriaxone,the genes isolated from HVS sample more sensitive to Framycetin and more resistant to Imipenem, Ceftriaxone and Ceftazidime, and genes isolated from wound swab more sensitive to Imipenem and Gentamicin and strong resistant to Ceftazidime.

Table 4. Show association of genes and antibiotic

Gene		Antibiotics No (%)															
		IMP		CN		AK		F		CRO		CIP		MEM		CAZ	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
AggRks	P	5 (10)	3 (6)	3 (6)	5 (10)	4 (8)	4 (8)	4 (8)	4 (8)	0 (0)	8 (16)	2 (4)	6 (12)	8 (16)	0 (0)	2 (4)	6 12%
	N	26 (52)	16 (32)	21 (42)	21 (42)	25 (50)	25 (50)	18 (36)	24 (48)	11 (22)	31 (62)	15 (30)	27 (54)	21 (42)	21 (42)	12 (24)	30 60%
Stx	P	2 (4)	0 (0)	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)	0 (0)	2 (4)	0 (0)	2 (4)	2 (4)	0 (0)	0 (0)	2 (4)
	N	29 (58)	19 (38)	23 (46)	25 (50)	28 (56)	20 (40)	21 (42)	27 (54)	11 (22)	37 (74)	17 (34)	31 (62)	27 (54)	21 (42)	14 (28)	34 (68)
IpaH	P	5 (10)	6 (12)	3 (6)	8 (16)	5 (10)	6 (12)	6 (12)	5 (10)	1 (2)	10 (20)	3 (6)	8 (16)	6 (12)	5 (10)	1 (2)	10 (20)
	N	26 (52)	13 (26)	21 (42)	18 (36)	24 (48)	15 (30)	16 (32)	23 (46)	10 (20)	29 (58)	14 (28)	25 (50)	23 (46)	16 (32)	13 (26)	26 (52)
SK	N	31 (62)	19 (38)	24 (48)	26 (52)	29 (58)	21 (42)	22 (44)	28 (56)	11 (22)	39 (78)	17 (34)	33 (66)	29 (58)	21 (42)	14 (28)	36 (72)

The AggRks ,Stx, IpaH genes are more sensitive to Imipenem and Meropnem and strong resistant to Ceftriaxone , Ciprofloxacin ,Ceftazidime and Gentamicin

4.6Detection of virulence factors of *E. coli* isolates in clinical specimens

Out of 50 isolates, 21 (42%) were positive for *IPaH, Stx* and *AggR* genes while 29 (58%) were negative for all these genes.*IPaH* gene was detected in 11 (22%), *AggR*gene was detected in 8 (16%) and stx gene was detected in 2(4%). Both *AggR*and *Stx* were co-existed in 2 (4%), both *AggR* and *IpaH*were co-existed in 3 (6%) and there was no *eae*gene detected in all isolates.

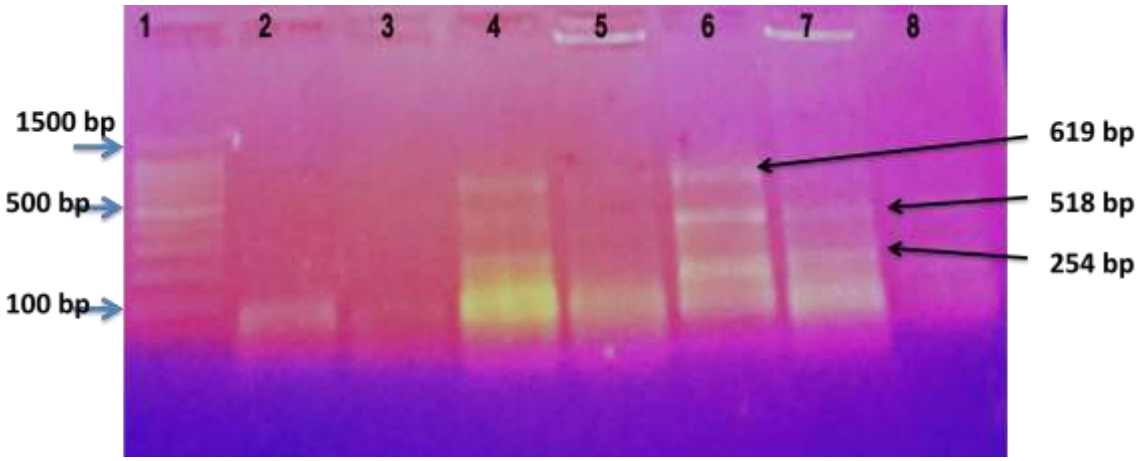

Figure 3: Multiplex PCR of *E.coli* strains using virulent specific primers. Lane 1: Gene ruler100bp; lane 2: negative control. Lane 3: Negative sample. Lane 4, 5, 6, 7 and 8 Multiplex of *eae* (EPEC), *ipaH* (EIEC), *stx* (EHEC), *aggR* (EAEC); *ipaH* (619bp); *stx* (518bp) and *aggR* (254bp).

4.6.1 Amplification of *aggRks* (254bp) target gene

A total of 50 *E. coli* isolates were identified from3 different samples (urine, wound swab and HVS),they were subjected to PCR analysis, only 16 isolates show band typical in size (254bp) indicated by the standard DNA marker (Table 5).

Table 5. The *aggRks* gene Frequencies in diarrheagenic *E.coli* isolates

Samples	*aggRks* gene	Frequencies	Percentage (%)	Total
Urine	Positive	8	(16)	40
	Negative	32	(64)	(80)
HVS	Positive	0	(0)	3
	Negative	3	(6)	(6)
Wound swab	Positive	0	(0)	7
	Negative	7	(14)	(14)
Total		50	100	50 (100)

aggRks detected in male and female in same percentage in 8(16%) , 4 male and 4 female Figure 3

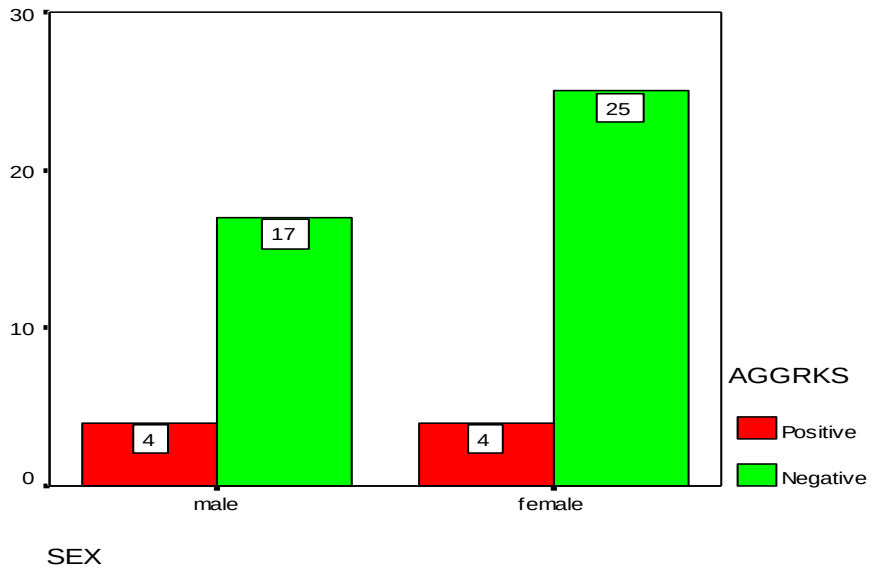

Figure 4 distributions of *aggRks* gene and sex

4.6.2 Amplification of*stx (*518bp) target gene

A total of 50 *E.coli* from 3 different samples (urine , wound swab and HVS) were subjected to PCR analysis , only 2 isolates showed band typical in size to 518bp indicated by the standard DNA marker (Table 6).

Table 6.*stx gene* Frequencies of diarrheagenic *E.coli* isolates

Samples	*stx gene*	Frequencies	Percentage (%)	Total
Urine	Positive	2	(4)	40 (80)
	Negative	38	(76)	
HVS	Positive	0	(0)	3 (6)
	Negative	3	(6)	
Wound swab	Positive	0	(0)	7 (14)
	Negative	7	(14)	

The *stx* gene found in male 2 (4%) of total 50 sample and not detected in female. Figure 5

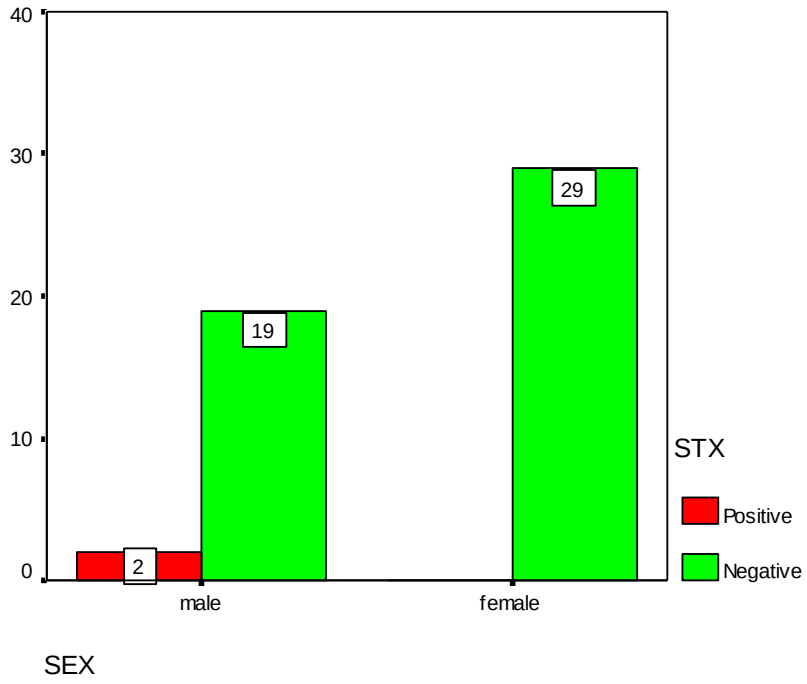

Figure 5 Distribution of *stx* gene according to sex

4.6.3 Amplification of *IpaH* (619bp) target gene

A total of 50 E.coli from 3 different samples (urine , wound swab and HVS) were subjected to PCR analysis , only 11 isolates show band typical in size (619) indicated by the standard DNA marker . (Table 7)

Table 7.*IpaH*gene Frequencies of *E.coli* isolates

Samples	IpaH gene	Frequencies	Percentage (%)	Total
Urine	Positive	8	(16)	40 (80)
	Negative	32	(64)	
HVS	Positive	1	(2)	3 (6)
	Negative	2	(4)	
Wound swab	Positive	2	(4)	7 (14)
	Negative	5	(10)	

The *IpaH* gene found in male more than female , positive in 11(22) samples, 6 male and 5 female of total 50 samples and the common detected gene in this study. Figure 6

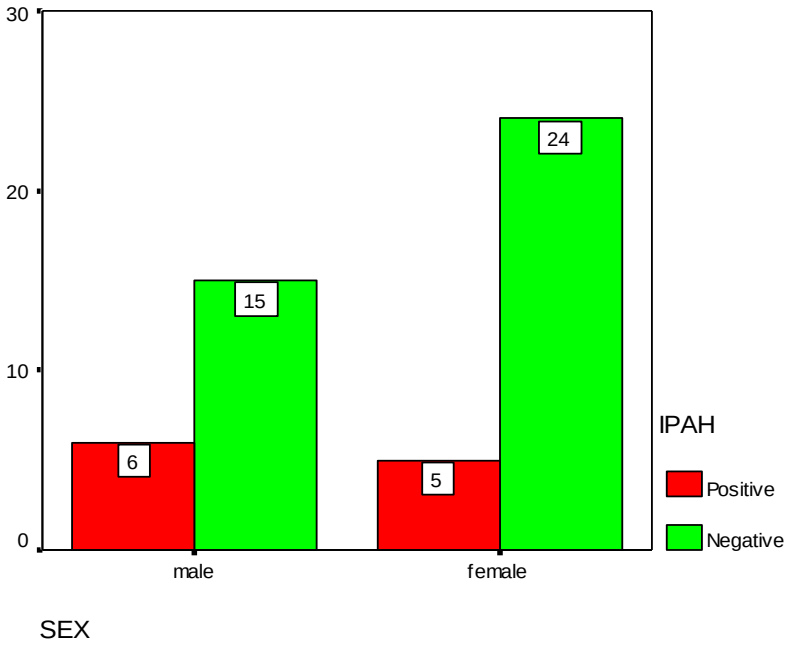

Figure 6 distribution of *IpaH* gene according to sex

4.6.4 Amplification of *eae* (881bp) target gene

A total of 50 *E. coli* from 3 different samples (urine, wound swab and HVS) were subjected to PCR analysis, all isolates no gives bands (Table 8).

Table 8.eae gene Frequencies of *E.coli* isolates

Samples	*eae* gene	Frequencies	Percentage (%)	Total
Urine	Positive	0	(0)	40 (80)
	Negative	40	(80)	
HVS	Positive	0	(0)	3 (6)
	Negative	3	(6)	
Wound swab	Positive	0	(0)	7 (14)
	Negative	7	(14)	

CHAPTER FIVE

DISCUSSION

Chapter Five

Discussion

5. Discussion

This research was aimed to detect the diarrheagenic *E.coli* virulence factors in samples other than stool, using of multiplex PCR. Which is sensitive and rapid method for detection and characterization. In the present study out of 50 isolates,29(58%) female and 21(42%) male 21 (42%) were positive for *IPaH*(8),*Stx* (2)and *AggR* (8) genes while 29 (58%) were negative for all these genes. Most of them from urine samples 86% (18/21), this may be due to the female genitourinary tract is more prone to bacterial infection due to nearing of these systems to rectal and leading to transmission of bacteria to these systems . (Dielubanza and Schaeffer, 2011).

Enteroivasive *E.coli* (*IpaH* gene) was most predominant DEC strain responsible for infection of urinary tract, genitourinary tract and wound that represented 22% of all isolate sample (16% in urine sample and 2% in HVS and 2% in wound swab), EIEC is important pathogens in developing countries where sanitation and hygiene levels have deteriorate (Rashedul., 2011), poor living conditions, inadequate water supplies, and insufficient education (Gomes *et al*,. 2016). Also the *IpaH* gene is responsible for invasion of urinary tract by attachment to the mucosa, which is facilitated by specific bacterial adhesions, which firmly connect the microbe to the tissue (Muenzne r*et al*., 2016).The presence of DEC in wound may be due to transmission of bacteria from contaminated hands to wounds.Enteroaggregative*E.coli* was represented 16% from all cases, all of them from urine; this finding is in disagreement with Saeed *et al*. (2015), who isolate the Enterohaemorrhagic*E. coli* represent (4%) from all cases found in urine sample. The isolated *E.coli* wasmore sensitive to imipenemand more resistant to; ceftriaxone 64%,

ceftazidime 56% ciprofloxacin 52% , framycetin 48% and Gentamicin 44% and few frequencies resistance toamikacin 34%, meropenem 30% and imipenem 28%, This finding is similar to Cao e*t al*.(2011).and little resistance to imipenem as study in (Moini *etal* ., 2015).that it is strong association between present of genes and antibiotic resistant of *E.coli* the most antibiotics resistant due to high consumption of antibiotic without return to doctor , irrational use, incomplete course of therapy, use of treatment without prescription from physicians. These make *E.coli* strong virulence factors to cause infections.

Conclusion & Recommendations

6.1 Conclusion

- From this study we concluded that, the presence of diarrheagenic *E.coli*in other samples than stools may indicate the source of infection may be contamination from feces.
- EIEC was most predominant in urine specimens.
- *E. coli* is more sensitive to Impenem and more resistant to ceftriaxne.

.6.2 Recommendations

1- In this study used of sample size of sample, we recommend using of large size and collected from different states of Sudan.

2- Use of more virulence factors gene to more confirm.

3- In this study the age of patient not include because not available.

We recommend including the age to give the association and presentation of gene in different age group.

4- Molecular technique other than PCR like sequencing and Restriction fragment length polymorphism (RFLP) should be used to confirm the pathotype of diarrheagenic*E. coli* strains.

5-Personal hygiene is important to protect from infectious disease.

6-Washing your hands before and after eating and doing anything.

7-Good health education to all population in the community.

8-to prevent antimicrobial resistance it should be don't used of inappropriate empiric antibioticheagenic .

9-prevent eating of food contaminated with Diarrheagenic *E.coli*especially under cooked beef such as hamburgers .

10-washing hands after touching animal at farm or petting zoos.

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APPENDIXES

Appendix I

1: Nutrient Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredients	g/L
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00

Preparation:

Suspend 28.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely.Sterilized by autoclaving at 15 Ibs pressure ($121°c$) for 15 minutes .pour into sterile Petri plates.

2: Eosin methylene blue (Himedia Laboratories Mumbai, India)

Ingredients	g/L
Peptone	10.000
Lactose	10.000
Dipotassuim hydrogen phosphate	2.000
Eosin yellow dye	0.400
Methylene blue dye	0.065
Agar	15.000

PH 6.8 ± 2

Suspend 37.5 g in 1000ml distilled water .bring the boil to dissolve completely .sterilize by autoclaving at 121◦C for 15 minutes . Cool to 60◦C and shake the medium in order to oxidase the methylene blue

(i.e.restore its blue colour) and to suspend the precipitate which is an essential part of the medium .

3: Kosser‘s citrate (mast group Ltd, Merseyside, uk).

Ingredients	Gms\litre
Magnesium sulphate	0.20
Potassium disulphate phosphate	1.00
Sodium ammonium phosphate	1.50
Tri sodium citrate	2.50
Bromothymole blue	0.016

Suspended by swirling 5.2grams of powder in litre DW mix well and sterile by autoclaving at 12-15 ibs pressure (118- 121°c) for 15 minutes

4: urea agar base (High media laboratories Pvt. 1td, India).

Ingredients	Gms|litre
Pepetic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Di sodium phosphate	1.20
Monopotassium phosphate	0.80
Phenol red	0.012
Agar	15.00

PH 6.8 at 25°c

24.0 grams in 950ml Dw sterile by autoclaving at 12-15 ibs pressure (118- 121°c) for 15 minutes ,cool to 50◦C and aseptically add 50ml of sterile 40% urea.

5: Peptone water (High media laboratories Pvt. 1td, India).

Ingredients	Gms|litre
Pepetic digest of animal tissue	10.00
Sodium chloride	5.00

PH 7.2 at 25°c

15.0 grams in 100ml Dw sterile by autoclaving at 12-15 ibs pressure (118- 121°c) for 15 minutes

6: Kiligler ironagar(High media laboratories pvt . 1td, India).

Ingredients	Gms|litre
Pepetic digest of animal tissue	15.00
Beef extract	3.00
Yeast extract	3.00
Proteose peptone	5.00
Lactose	10.00
Dextrose	1.00
Ferrous sulphate	0.20
Sodium chloride	5.00
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	15.00

PH 7.4 at 25°c

7: Muller Hinton agar (High media laboratories pvt . 1td, India).

Ingredients	Gms\litre

Beef infusion	300.00
Casein acid hydrolyate	17.50
Starch	1.50
Agar	17.00

PH 7.3 at 25 °*c*

Suspend 38.15 grams in 1000ml Dw sterile by autoclaving at 12-15 ibs pressure (118- 121°*c*) for 15 minutes, and pour into sterile petriplates.

8 : Kovac´s Reagent (High media laboratories pvt . 1td, India).

Ingredients	g/L
p- dimethyl amino benzaldehyde	10g
isoamyl alcohol	150 ml
concentrated hydrochloric acid	50ml

preparation

Kovac´sreagentis prepared by dissolving of 10gm of p- dimethyl amino benzaldehyde in 150 isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid .

Appendix II: antibiotic discs

Imipenem	IPM	10 mcg/disc
Ceftriaxone	CRO	30 mcg/disc
Meropenem	MEM	10 mcg/disc
Amikacin	AK	30 mcg/disc
Ciprofloxacin	CIP	5 mcg/disc
Ceftazidime	CAZ	30 mcg/disc
Gentamicin	CN	10 mcg/disc
Framycetin	F	100 mcg/disc

Reagent

1- Hydrochloric acid 1mol|L

Concentrated Hydrochloric acid 8.6 ml and 100ml distilled water

2- Kovac‘s reagent

(p) – di methyl aminobenzaldehyde 2 gram

4- Macforland standard

Sulphuric acid solution 99.5ml

Barium chloride solution 0.5ml

5- Normal saline (phisological saline)

Sodium chloride 25gram in 1 liture distilled water .

***The modele of incubator**

GALL Enk AMP GE made in UK

***The modele of Autoclave**

Griffin and Italy George 1td

***The modele of Hot air oven**

Leader made in England

***The modele of Water bath**

Memert cmb1t co.KG, England

*** The modele of Microscope**

Olympus optical .LTD,UK

*** the modele of Centrifuge**

Hettich , Germany

***The modele of Refrigerator**

Starlet Europe , Europe

***The modelePCRthermo cycler**

Teche Tc-312, UK

***The modele of Electrophoresis apparatus**

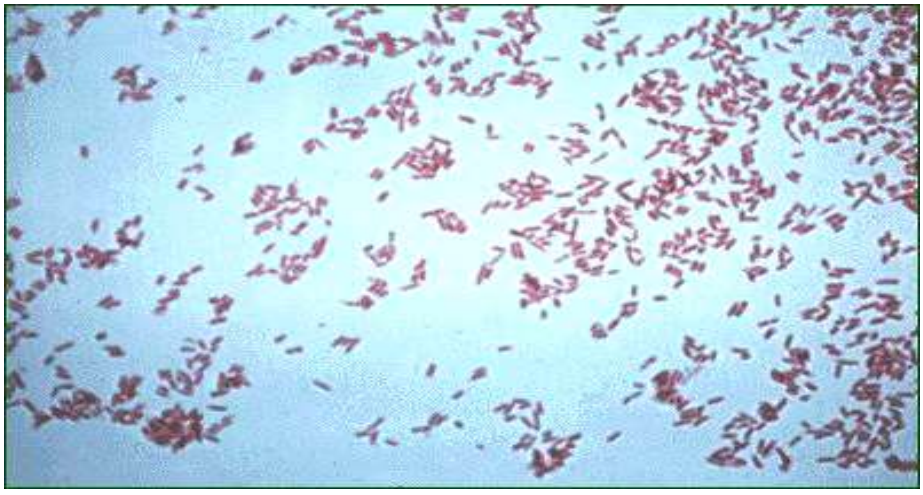
Primer ,125V,500MA,UK

***The modele UV transilluminator**

Uvite –UK

Appendix III:

1: Gram stain of *E.coli*



2: E.coli in EMB media

3: sensitive balance

4: PCR Machine

5: Centrifuge

6: Microwave

7: Gelelectrophoresis Device

Appendix IV

Sudan University of Science and Technology

College of Graduate studies

Questionnaire

Molecular Detection and Characterization of Diarrheagenic *Escherichia coli* from Clinical isolates in Khartoum State

By:

Athar Hassan Mohammed Elyas

Date: ……

No:……

Type of specimen:……

Name…….

sex: ……

Resident …….

Symptoms: …….