Sudan University of Science and Technology
College of Graduate Studies

Detection of Virulence Genes of DiarrheagenicEscherichia coli Strains, Isolated fromDrinking Water in Khartoum State

الكشف عن الجينات الممرضه لبكتريا الإشريكية القولونيه المسببة للإسهال المعزولة من مياه الشرب في ولاية الخرطوم

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قال الأمام الشافعي:

"طلبنا العلم لغير الله فأبى العلم إلا أن

يكون لله"

DEDICATION

I dedicate this work to:

My father, bless upon him.

My mother who has been my constant source of support and love.

My sisters, brothers, and my friends whom helped me in my life and gave me the force to continue.

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Abstract

Diarrheagenic *E. coli* (DEC) strains are an important cause of intestinal syndromes in developing countries mainly affecting children, DEC strains are typically transmitted by contaminated food and water. This study was aimed to determine the prevalence of virulence genes in the diarrheagenic *E. coli* (DEC) isolated from drinking water in Khartoum State from April to May 2016.

A total of 46 isolates of *E. coli* were obtained from Central Public Health Lab in Khartoum. These isolates were isolated from drinking water in Khartoum State and subcultures in Eosin Methylene Blue (EMB) media, the isolates were then identified by routine biochemical tests. Antimicrobial susceptibility testing was then done by using the following antibiotics; Chloramphenicol, Ceftriaxone, Ciprofloxacin, Gentamicin, and Tetracycline. Bacterial genomic DNA was extracted by boiling centrifugation method, and Multiplex Polymerase Chain Reaction (MPCR) was performed to detect the virulence genes (*IPaH, Stx, AggR*, and *eae*). Most of the isolated bacteria were from tap water 86.9% (40/46), and the rest from cooler 10.9% (5/46) and tanks 2.2% (1/46). In addition to, the majority of them were from houses 36 (78.3%), most of them in Khartoum province 20(43.5%). Most of the isolates were resistant to Tetracycline 45(97.8%), 8 (17.4%) were resistant to Chloramphenicol, 4 (87.0%) were resistant to Ceftriaxone, 11 (23.9%) were resistant to Ciprofloxacin and 11 (23.9%) were resistant to Gentamicin.

The multiplex PCR assays confirmed the presence of *E. coli* virulence genes in 58.6% (27/46) of all the isolates; IPaH gene 41.3% (19/46), *AggR* gene 30.4% (14/46) and *Stx* gene 10 .8% (5/46). There were 9(19.5%) isolates positive for both *AggR* and *IPaH*. Also, *AggR* and *Stx* genes were detected both in 2(4.3%) isolates. All isolates were negative for *eae* (EPEC). There were 19 (41.4%) negative for all genes.

This study confirmed for the first time the presence of *DEC* strains in drinking water in Khartoum State in different resources and different locations. Also, Detection of diarrheagenic *E. coli* by Multiplex PCR method was quite satisfactory. We recommended that the drinking water should be treated before use and also, using of the filters in tap's spouts of a great importance.

المستخلص

سلالات الاشيركية القولونية المسببة للاسهال هى من اهم مسببات الاضطرابات المعوية فى البلدان النامية والاطفال هم اكثر تأثرا بها وهى تنتقل عن طريق الطعام والشراب الملوثين , هدفت هذه الدراسة لتحديد أنتشار العوامل الممرضة (الجينات) في الإشريكية القولونية المسببة للإسهال في مياة الشرب بولاية الخرطوم في الفترة ما بين أبريل- مايو 2016م. وقد شملت الدراسة 46 من الإشريكية القولونية المعزولة من مياه الشرب في المعمل القومي المركزي في ولاية الخرطوم. وتم التعرف عليها بتزيعها في الوسط الزراعي EMB واجراء الإختبارات الكيموحيوية الروتينية. وأجري لها ايضا أختبار الإستجابة للمضادات الحيوية بأستخدام المضادات الحيوية التالية: الكلورامفينكول، السيفترايكزون، السبروفلاكسين، الجنتاميسين والتتراسايكلين.

الجينوم البكتيري أستخلص بطريقة الغليان والطرد المركزي، وأستخدمت طريقة تفاعل البلمرة المتسلسلة المتعددة (mPCR) في تحديد الجينات الممرضه (*eae, AggR Stx IPaH*). أغلب البكتيريا المعزولة كانت من مياة الحنفيات % 86.9 (36/46) والباقي من المبردات % 10.9 (5/46) والخزانات % 2.2 (1/46). بالإضافة لهذا فأن معظم البكتريا المعزولة كانت من المنازل % 87.8 (36/46) من محلية الخرطوم % 43.5 (20/46). المقاومة للتترسايكلين سجلت في 45 (% 97.8) عينة والكلورامفنيكول 8 (% 17.4) 4 (97.0%) والسيفترايكزون و 11 (% 23.9) عينة كانت مقاومة لكل من السبروفلاكسين والجنتامايسين.

أختبار تفاعل البلمرة المتسلسلة المتعدد أكد وجود الجينات الممرضة للإشريكية القولوقونية في % 58.6 (27/46) من المجموع الكلي للعينات المعزولة، حيث كانت نسبة الجين *IPaH* 41.3% (19/46) و الجين *AggR* 30.4% (14/46) و الجين *Stx* 10.8% (5/46). وجدنا أن هنالك 9 (19.5%) عزلات كانت موجبة للجينين *AggR* و *IPaH* وأيضاً الجينين *AggR*, *Stx* وجدا مشتركين في 2 (4.3%) من العينات. كل العينات المعزولة كانت سالبة للجين *eae* (46/46) و 19 (41.3%) كانت سالبة لكل الجينات.

هذه الدراسة أكدت للمرة الأولى على وجود سلالات الإشريكية المسببة للإسهال في مياه الشرب بولاية الخرطوم من مصادر وأماكن مختلفة. كما أن الكشف عن الإشريكية القولونية المسببة لإسهال عن طريق تفاعل البلمرة المتسلسلة المتعدد كان فعالا. نوصي بضرورة معالجة مياه الشرب قبل الإستعمال مع أستخدام المرشحات في صنابير المياه.

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Abbreviations

TMP Trimethoprim

SMXSulfamethxazole

KIAKligler's Iron Agar

DNADeoxyriboseNucleic Acid

EDTA Ethylene DiamineTetraacetic Acid

TBETrisborat/Boric Acid/EDTA buffer

WHO World Health Organization

ELISA Enzyme- Linked Immune Sorbent Assay

CFA I Colonization factor antigen I

CFA II Colonization factor antigen II

LPS Lipopolysaccharide

DW Distilled water

HIV Human Immunodeficiency Virus

Chapter One

Introduction

1-Introduction

1-1 Introduction

Good health depends on a clean, drinkable water supply. This means that water must be free of pathogens, dissolved toxins and disagreeable turbidity, color and taste (Cowan and Herzoge, 2014). Through ordinary exposure to air, soil and effluentsurface water usually acquire harmless, saprobic microorganisms. But also can pick up pathogenic contaminants "waterborne pathogens" like protozoa (*Gardia* and *Cryptosporidium*), the bacteria (*Campylobacter*, *Salmonella*, *Shigella* and *Vibrio*) and Hepatitis A and Norwalk viruses (Cowan and Herzoge, 2014).

The determination that the water is consequently unsafe to drink is by focusing on detecting fecal contamination "high fecal levels can mean that water contains pathogens and other water sources can be analyzed for the presence of various indicator bacteria. In the late 1800s, it was suggested that the best way to determine if water or its products has been exposed to feces, is by testing for the presence of *E. coli* (Cowan and Herzoge, 2014).

Escherichia coli (*E. coli*), it commonly found in thegastrointestinal tract of humans as normal flora and in a wide range of other animals. This bacterium can be shed, often in high number via the faeces into the environment (WHO, 2013). Most strains of *E. coli* are harmless, most human beings have a significant concentration of such beneficial *E. coli* in their gut "up to 1.000.000 per gram of faeces" (WHO, 2013). Some *E. coli* strains, however, can cause disease. For exampleEnterotoxogenic*E. coli*(ETEC) strains produce a toxin in the gut. Resulting typically in diarrhea and can lead to serious disease. And other strains can cause life–threatening–disease for exampleEnterohaemorrhagic*E. coli*((EHEC) can cause Hemolytic Uremia Syndrome (HUS). (WHO,2013). *E. coli* is Genus in Enterobactericeae family, Gram – negative rod shaped bacterium, widely distributed in nature, facultative anaerobes, motile by peritrichous flagella

with ability to ferment lactose rapidly,usualy within 24-48 hours, lack of cytochrome C oxidase and catalase positive (Ryan *et al.*, 2010).

E. coli is classified in the, phylum, proteobacteria, class, Gammaprotebacteria, Family,Enterobactericeae, Genus, *Eschericha*, species, *E. coli* (Todar, 2008).

Constitute part of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacteria cause disease (Todar, 2008).

Pathogenic *E. coli* are classified in distinct categories according to their virulence properties, symptoms of disease that they cause, species and age group where they are found (Brooks *et al.*, 2010).These criteria have been the basis for the division of human diarrheagenic *E. coli* "DEC", in at least five categories, Enteropathogenic*E. coli* "EPEC", Enterotoxogenic*E. coli* "ETEC", Enteroinvasive*E. coli* "EIEC", Enterohemorrhage*E. coli* "EHEC" and Enteroaggareagtive*E. coli* "EAEC" (Ryan *et al.*, 2010).

The commonly used diagnostic tests for *E. coli* disease are Gram stain for rod Gram-negative, culture of specimens in selective and differential media such as MacConkey and Eosin Mythelen Blue(EMB) media, by biochemical tests such as indole test, Serology test to detect the toxin product and ELISA. *E. coli* strains that cause diarrhea can be differentiated base on tests of DNA probe and Polymerase Chain Reactions "PCR" (Brooks *et al.*, 2010).

DECis among the most common etiologic agents of diarrhea from water origin(Fakhr*etal*., 2016). Fecal contamination of drinking water by DEC is a major health problem which accounts for many cases of diarrhea mainly in infants and travelers,more than 800,000 children younger than 5 years of age die from diarrhea mostly in developing countries (Jafair*etal*.,2012).Antibiotic resistance among bacterial isolates complicate the problems because *E. coli* can be considered as an important vehicle for the spread of resistance genes due to its plenty in such environments with a high risk of transfer fromthe environment to human (Ozgumus*etal*.,2007).

1-2Rationale

Most drinking water comes from rivers and wells, and used in its natural form. In most cities, it must be treated to be clean and free of contaminants before itsdistribution to consumers. In many parts of the world, Sewage water draining out the homes and industries that contain a wide variety of chemicals, debris and microorganisms and can contaminate drinking water (Cowan and Herzoge, 2014). The importance of safe water is that there are waterborne microorganisms responsible for worldwide outbreaks e.g. “Cholera” which have killed thousands of people. Water–related disease cause 3.4 mullions death every year, diarrheal disease affect mainly children in developing countries, and 88% of the reasons are due to unsafe water supply, sanitation and hygiene (WHO, 2014b).

In recent years, KhartoumState witnessed a large movement of people from the peripheral states to Khartoum State, which makes it a big community characterized by crowding, poor sanitation and inadequate water supply.Diarrheal and gastroenteritis were the fifth leading causes ofhospitals admission in Khartoum State in 2015(Sudan Annual Statistical Report, 2015).Becauseof there is noany previous study about detection of DEC in drinking water, weconducted thisstudy to determine the presence of DECvirulent genes in drinking water in Khartoum State.

1-3 Objectives of the study

1.3.1General Objective

To determine the presence ofdiarrheagenic*E. coli*(DEC) strains (EHEC, EPEC, EIEC and EAEC) in drinking water in Khartoum State, Sudan.

1.3.2. Specific Objective

1 .To determine the most probable contaminated water source in Khartoum State.

2. To find out antimicrobials sensitivity patterns of isolated strains.

3. To determine the presence and prevalence of the virulence factors;*Stx*(EHEC),*eae*(EPEC), *IPaH*(EIEC) and *aggR*(EAEC) of DECstrainsin drinking water in Khartoum State.

4.To determine the reliability of the Multiplex PCR as quick diagnostic procedure for detection ofDiarrheagenic*E. coli* strains (EHEC, EPEC, EIEC and EAEC).

Chapter Two

Literature Review

2. Literature Review

2.1 Background

Water covers over 70% of the Earth's surface and it is a very important resource for human and the environment. Clean, safe, and adequate freshwater is vital to the survival of all living organisms. A wide range of human and natural processes affect the characteristic of water and make water unsuitable for human use (Palaniappan*et al.*, 2010). Water pollution means contamination of water with physical, chemical or biological agents which have effects on human life. The main source of water pollution is home waste water and dirty municipal waste, sewage (Chamaria, 2015).

Waterborne diseases are caused by pathogenic microorganisms that are transmitted in drinking contaminated or dirty water. Water borne disease can be spread via ground water, which is contaminated with trench latrines causing of many types of diarrheal diseases, including Cholera and other serious illnesses such as typhoid and dysentery (WHO, 2014a).

2.2 Diarrheal Diseases

Diarrhea is defined as the passage of three or more loose or liquid stools per day "or more frequent passage than in normal for the individual" (WHO, 2013).Diarrhea is usually a symptom of an infection in the gut, which can be caused by different types of bacterial, viral and parasites.Infection is transmitted via contaminated food or drinking water, or from person to person as a result of poor hygiene (WHO, 2013). Diarrheal disease was the third leading cause of death in low – income countries, causing 6.9% death overall, and it is the second leading cause of death in children under five years old, and children under three years old experience on average three times of diarrhea per a year (WHO, 2014b).Diarrhea, can last over several days, and can leave the body without the water and salt. Most people who die from diarrhea actually die from severe dehydration and fluids loss. Children who are malnourished or immune compromise as well as people with HIV are most risk

of life–threatening diarrhea (WHO, 2013) in other wise many patients with diarrhea need no treatment because the disease is usually self-limiting (Cheesbrough, 1995).

2.3 Etiology

E. coli are members of a large group of bacterial germ that lives in the intestinal tract of humans and other worm-blooded animals "mammals, birds", new borne have sterile alimentary tract which within two days becomes colonized with *E. coli* (Marler, 2011). Most strain of *E. coli* is harmless and is important to good digestive health and provides their hosts with vitamin K. (Todar, 2008). Some *E. coli* strains, can cause disease are referred to as pathogenic strains, are harmful and produce toxins (e.g. EIEC) and some strains can cause very serious–and in some cases life-threatining disease (e.g. EHEC)(WHO, 2014c) including specifically *E. coli* 0157:H7

2.4 General characteristic:

*E. coli*is a rod–shaped bacterium, each bacterium measures approximately 0.5 μm in width and 2μm in length? *E. coli* is a Gram negative bacterium; the *E. coli* cells stain Gram negative because they have a thin cell wall with only 1-2 layers of peptidoglycans. *E. coli* is facultative anaerobic, grow better in the presence of oxygen,non- spore forming, motile by peritrichous flagella, has the ability to ferment lactose rapidly within (24 hours) (Parija, 2009). Producing acid and gas, catalase positive, oxidase citrate and urease are negative (Baylis *et al.*, 2011). The bacterium can grow and cultured easily and inexpensively in the laboratory. *E. coli* is chemoheterotrph, cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination (Pommerville, 2007).

2.5**GenomesStructure:**

The first complete DNA sequence of an *E. coli* genome was published in 1997.It was found to be a circular DNA molecule 4.6 million base pairs in length,containing 4288 annotatedprotein-coding genes ,seven ribosomalRNA(rRNA)operons,and 86 transfer RNA(tRNA) genes.The coding density was found to be very high,with a mean distance between genes of only118 basepairs.The genome was observed to contain a significant number of transposable genetic elements,repeat elements ,cryptic prophages,and bacteriophage remnants(Blattner*etal*,1997)

2.6 Taxonomy

E. coli is a member of the Domain: Bacteria, Kindom: Bacteria, Phylum: Proteo bacteria, class Gammaproteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Escherichia*, Species: *E. coli (E. coli*) (Todar, 2008).

2.7 Antigenic Structure

E. coli types are based on major surface Antigens:

O-antigen: Antigen is part of the lipoplysaccharide, OAg "somatic antigen" is heat stable, detected in agglutination assays with specific rabbit antibodies. Hundreds of O groups have been identified.

H-antigen: Antigen is flagellin protein, heat labile, it weakens chemical agent "Alcohol" or weak solvent to extract it.

K-antigen: a-antigens were named for capsule (Kapsel in German), K Antigens overlay the surface O-Ag, heat stable (Parija, 2009). Sera subdivided K-antigens into L, B and A groups, at the molecular level, there are two major of types of K antigens

Polysaccharid K antigens

Protein Antigens mostly fimbriae (Davies, 2014).

More than 700 serotypes of *E. coli* have been classified by sharingOAg and HAg that define serotypes, and define to sero groups (OAg only) (Marler,

2011). The *E. coli* O groups have shown remarkable cross–reactivity with O antigens from other members of the Enterobacteriaceae, mostly in is *Shigella*spp (Mahan *et al.*, 2014).

Fimbrial Antigens: - Exhibit fimirate and strains may carry both sex pili and more than one type of fimbrial structure adhesion to a wide range of human and animal cells that contain the sugar mannose, the adhesion might be involved in pathogencity cause mannose – resistant hemagglutination and they play an important role in the pathogenicity of diarrhea disease and urinary tract infection (UTI). Fimbriae are long, threadlike protein polymers found on the surface of many strains of *E. coli* heat labile protein. Fimbriae of *E. coli* are strongly immunogenic (Davies, 2012).

2.8 Virulence Factors

Nowadays, *E. coli* particularly diarrheagenic strains are classified depending on their unique virulence factors (Todar, 2007). Pathogenic *E. coli* strains use multistep system of pathogenesis that is same to that is used by other mucosal pathogen, which consists of the colonization of the f mucosal site, evasion of hosted defiance, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extra cellular, but (EIEC) is a true intracellular pathogen that is can invade and replicating within epithelial cells and macrophages (Kaper*et al.*, 2004). There are summarized of the virulence factors determinate of pathogenic *E. coli* (Todar, 2007).

Adhesions	CFA1/ CFA11/ Type p fimbriae/ P fimbriae innteimin(non-fimbrial adhesion EPEC adherence factor.
Invasions	Hemolysin/ Shigella –like ivasions(for intracellular invasion and spraed).
Toxins	LT toxin/ ST toxin/ Shiga toxin/ Cyto toxin/ Endotoxin LPS.
Antiphagocytic surface properties	Capsules/ KAgs/ LPS.
Defense against serum bacterial reaction	LPS/ KAgs.
Defense against immune response	Capsules/ KAgs/ LPS/ Antigenic variation.
Motility/ Chemotaxic	Flagella

E. coli consists of a different group of bacteria, pathogenic E. coli strains are classified into six patho types, which are associated with diarrhea and are named to as diarrheaogenic*E. coli*.

Shigatoxin – producing *E. coli* (STEC) may also be named to as verocytotoxin–producting*E. coli* (VTEC) or Enterohemorrhagic *E. coli* (EHEC) this pathotype is the one most known heard about its associated with food borne outbreak (Ryan *et al.*, 2013).

Enterotoxigenic*E. coli* (ETEC).

Enteropathogenic *E. coli* (EPEC)

Enteroaggregative *E. coli* (EAEC).

Enteroinvasive *E. coli* (EIEC).

Diffusely adherent *E. coli*(DAEC).

2.9 Epidemiology

Travel to developing countries is associated with higher risks for traveler's diarrhea, ETEC is the most common pathotype that cause diarrhea among travelers returning from most regions, other pathotypes can also cause traveler's diarrhea, Travel-associated infection caused by non STEC diarrheagenic *E. coli* are under recognized because most clinical laboratories do not use method that can detect them (Mody and O'Reilly, 2015).

Human is the main reservoir for strains causing diarrhea in people. The intestinal tracts of animal, especially cattle and other ruminates, are the primarly reservoirs of STEC (Kaper *et al.*, 2004).

2.10 Transmission:

Diarrheagenicpathotypes of *E. coli* can be transmitted from several sources:

- Food borne transmission, of Stx-producing *E. coli*in 1982 during an investigation into an outbreak of hemorrhagic colitis, the *E. coli* O157: H7 was first recognized as a food borne pathogen.

- Water borne transmission, water used for recreation (e.g. pools shallow, lakes) and from human consumption can also become contaminated.
- Animal to person transmission of *E. coli,* it had been identified in many outbreak stations as well as in isolated settings.
- Person to person transmission of*E. coli*. By *E. coli* 0157 : H7, which has occurred in day-care centers, hospitals, nursing homes and private residences, because the infectious dose is so small it is very easy for the bacteria to be transferred through people with close physical contact (WHO, 2016).

2.11 Clinical Infections

E .coli strains are one of the most frequent causes of several common intestinal and extraintestinal bacterial infections, by means of virulence factors that affect a wide range of cellular processes (Kaper*et al,* 2004).

Three general clinical syndromes can result from infection with one of *E. coli*pathotypes, enteric/ diarrheal disease,urinary tract infection *(*and sepsis/meningitis. Among the intestinal pathogens there are six–well–described categories: EPEC/ EHEC / ETEC, EIEC, EAEC and DAEC (Cheesbrough, 1995).

2.12.Pathogensandpathogencitiy2.12.1Enteropathogenic*E. coli* (EPEC)

It was the first pathotype of *E. coli* to be described, by Bray in 1945 in the United Kingdom in large outbreaks of infant diarrhea. EPEC remains an important cause of potentially fatal infant diarrhea in developing countries (Kaper*et al.,* 2004). EPEC strain usualy perform localized adherence (LA) to epithelial host cells and include attaching and effacing (A/E) lesions on enterocytes. EPEC virulence genes *eaf* and *eae* are respectively associated with LA and A/E lesions (Ogata et al., 2001). The EPEC pathotype can be classified into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC), which both they have the ability to cause A/E lesions. The human is the only

reservoirs for tEPEC, they are transmitted by inter-human contact. The tEPEC cause either watery or bloody diarrhea and during infections, EPEC bacteria adhere to intestinal epithelial cells and form a ctinfrich pedestal which is also a common feature of some EHEC strains (Baylis *et al.*, 2011). While aEPEC, human and a animals are the reservoirs found in developed countries as well as developing countries.

2.12.2Eenterohaemorrhagic*E. coli*(EHEC)

First recognized as a cause of human disease in 1982, the main reservoir of EHEC is healthy cattle intestinal tract and initial outbreaks were associated with consumption of under cooked burgers, other foodborne source, including sausages, unpasteurized milk, lettuce, apple juice and radish sprouts, it's loss have been associated with disease (Kaper *et al.*, 2004). EHEC cause bloody diarrhea (bloody diarrhea "hemorrhagic colitis", Non –bloody diarrhea and HUS as very serious and life-threatening condition for young children and the elderly (Pommerville, 2007) low infectious dose required for infection (estimated to be < 100 cells), (Ryan *et al.*,2010). EHECcan also transmit by recreational and municipal drinking water, person to person, petting zoo and farm visitations and recent report indicates no potential airborne transmission after exposure to contaminated building (Varma*et al*., 2003). EHEC strains of the O157: H7 serotypes are the most important EHEC pathogen, the key virulence factor for EHEC is Stx which also known as verocytooxcin (VT) because its effect the verocell, also named shigatoxin*(Stx)* because their same action of shigatoxin from *Shigella dysenteriae*(Baylis*et al.*, 2011).In addition to *Stx*, most EHEC strains also contain the LEE (locus of enterocytes effacement) pathogenicity island that encode a type III secretion system and effector proteins. More than 200 serotypes of *E. coli* have*Stx* and most of these serotypes doesn't contain LEE pathogenicity island and are not associated with human disease. This has led to the use of shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC) as general terms for any *E. coli* strains

that produce *Stx* and the term EHEC for the subset of *Stx* – positive strains that also contain the LEE (Kaper *et al.*, 2004).

2.12.3Enterotoxogenic*E. coli*(ETEC)

Is a major pathogen of diarrhea in young children in developing countries and in adult from developed countries traveling to these regions (Traveler's diarrhea) (Kaper *et al.*, 2004) ETEC through the fecal-oral route via contaminated foods and drinking water for humans and animal's infection. (Hill, 2013), A characteristic feature also cause diarrhea in new borne animals (calves, lambs).ETECtransmitted of ETEC is their ability to produce one or more heat stable (ST) or heat-labile (LT) enterotoxin. Infection by ETEC is because watery diarrhea with little or no fever due to use contaminated foods, raw vegetable and soft cheeses, there has been sporadic waterborne outbreak causes by ETEC (Baylis *et al.*, 2011).

2.12.4Enteroinvaisve*E. coli*(*EIEC*)

EIEC are biochemically, genetically and pathogenically closely related to Shigella spp. EIEC isdifferentiated from*Shigella* by a few minor biochemical tests, but they share essential virulence factors (Ryan *et al.*, 2010).

EIEC cause an invasive, dysenteric from of diarrhea in humans and infected human only, no known animal reservoir for EIEC. Infections occur via the faecal-oral route with contaminated soft cheese, potato salad and guacamole by infected (carrier) food handlers. Person to person transmission has also been reported (Baylis *et al.*, 2011).The site of infection is the colon and the ability of EIEC to invade and damage colonic tissue is associated with genes such as the invasion plasmid antigens *IPaA* to *IPaH* (Hill, 2013).

Common symptoms of infection include watery diarrhea that may precede Dysenteric stools with blood and mucus. Ulceration of the bowel can occur in severe illness (O'Sullivan *et al.*, 2006).

2.12.5Enteroaggregative*E. coli*(EAEC)

EAEC is another diarrheagenicpathotype that is primarly found in children in the developing world, and in the developed world in adults with HIV, travelers and as an occasional agent of food-borne outbreak.The infection feature by adherence to epithelial cells in a stacked brick-like aggregate within bioflim (Kaper *et al.*, 2004) infection is typically followed by a watery, mucoid, diarrheal illness with little to no fever, no vomiting (Hill, 2013).Aggregative adherence in EAEC is medicated by either aggregative adherence fimbrae (AAF/I) or (AAF/II) which are encoded forby aggR genes also EAEC had heat – stable toxin similar to ST and may be responsible for the symptoms of infection (O'Sullivan *et al.*, 2006).

2.12.6Diffuselyadherent*E. coli(DAEC)*

Is featured by a diffuse pattern of adherence to HEP 2 cells monocytes (Baylis *et al.*, 2011),similar to those EAEC strains. DAEC have been implicated as a cause of diarrhea in children >12 months age (Kaper*et al*2004)

2.13 Laboratory Diagnosis

2.13.1 Gram Stain

Gram staining is a common technique to differentiate the bacteria in two large groups based on their different cell wall contents (Bruckner, 2012). Microscopy *E. coli* is aGram negative rod, usually motile.

2.13.2 Culture

E. coli is anaerobic and facultative anaerobic it produce 1-4 mm in diameter colonies on blood agar after overnight incubation at 35□C – 37□C, the colonies may appear mucoid and some strains are hemolytic. On MacConkey agar most *E. coli* strains produce lactose fermenting colonies "deep red colonies", on EMB agar produce black colonies with green, black metallic sheen (Ryan *etal*., 2010).

2.13.3 Biochemical test

The basic biochemical reaction of *E. coli* is Indole positive (+), Urease negative (-), Citrate negative (-), KIA slope media is yellow Butt, yellow slant with gas and no H_2s, an important biochemical feature of most *E.coli* strains areIndole production of peptone water media containing tryptophan (Cheesbrough, 1995).

2.13.4 Serology:

H and O antigens have been identified for many of *E. coli*serogroups in the patient's sera where the pathogen cannot be cultured or where a rapid answer is needed (VTEC) *(*O'Sullivan *et al.,* 2006*).*

2.13.5 Histopathology

In tissue/ tissue culture to define a specific pathotype of diarrhea causing by EPEC exhibit atypical A/E lesion in intestinal biopsy specimens (Mims *et al.*, 2004).

2.13.6 Molecular methods

A number of nucleic acid based method, have been established for the detection and characterization of *E. coli* strains, the most commonly method is based on the use of Polymerase Chain Reaction (PCR) to amplify a specific gene target in *E. coli* strains. PCR may detect a characteristic virulence factor like (Vtx) in VTEC or *(eae) gen* in EPEC/EHEC (O'Sullivan *et al.,* 2006).

2.13.7 Typing of *E. coli* strains

Is essential in surveillance and epidemiology of infection, particularly in outbreak investigation of (VTEC) also typing to determine the pathogenicity and the virulence capacity of *E. coli* strains which is essential for control measurement and risk based legislation (O'Sullivan *et al.,* 2006).

2.14*E. coli* and coliform

In the late 1800s, it was suggested that a good way to determine if water or its products had been contaminated with feces was to test for *E. coli*, because most *E. coli* strains are not pathogenic, they almost always come from mammal's

intestinal tract, so their presence in a sample is a direct indicator of fecal contamination (Cowan and Herzoge, 2014)

At the time it was too difficult to distinguish *E. coli* from the closely related species of *Citrobacter*, *Klebsiella* and *Enterobacter*, Laboratories instead simply reported whether a sample contained one of these isolates "All are phenotypically similar". The terminology adopted was coliform –positive or coliform negative "coli form means *E. coli* like". Coliform, areGram-negative, lactose – fermenting, gas-producing bacteria (Ashbolt*et al.*, 2001).

2.15 Treatment

Because most of *E. coli* diarrheas are mild and self-limiting, treatment is usually not an issue. Rehydration and supportive care are the mainstay of treatment (Cheesorough, 1995).

Treatment with TMP- SMX or fluoroquinolones reduces the duration of diarrhea in ETEC, ELEC and EPEC infections (Ryan *et al.*, 2010).

In serious *E. coli* infection (HUS), the patient will be hospitalized and given supportive care, including fluids, blood transfusions and kidney dialysis (Greenwood *et al.*, 2002).

2.16 Vaccination

Scientists have managed to collect detailed information about the *E. coli* bacteria, which cause millions of cases of diarrhea a year, they found that strains of the *E. coli* cluster into surprisingly closely-related group this means the bacteria causing disease in South America are very similar to those in Africa and Asia. These similarities between these strains of bacteria mean a global vaccine is possible (Davies, 2014).

Beginning in 2009, commercial vaccine has been available to beef producers to significantly reduce *E. coli* in cattle digestive system by 50-75 percentage on average, with some cattle showing reductions as high as 98 percent. In 2013 a study on the vaccine estimated that their use could reduce *E. coli* infection in human by up to 86 percent. But beef producers have been slow to adopt the

vaccine. The most likely reason is that, given their cost, there isn't a clear enough economic (Andrews, 2015).There is no available vaccine for *E. coli* infections, and research continues on vaccine for all diarrheagenic *E. coli* strains.

2.17 Prevention

Food and water are the primary source of *E. coli* infection, so travelers should be:

Eating only cooked foods.Avoiding uncertain water, ice, salad and raw vegetables when travelling in developing countries.Consume only pasteurized milk and milk productsWashing fruits and vegetables before use.Washing fruits and vegetables before use.Washing hands after restroom with water and soap.Pasteurized requirement to fruit juice and cooked meat.Clinician should a educate people traveling to affected areas about the possibility of infection amongreturningravelers(Ryan*etal*,2010).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Study design

This was a descriptive cross-sectional study

3.2. Study area

The study was conducted at Central Public Health Laboratory at Khartoum State (Sudan).

3.3. Study Period

The study was carried out during the period from April to May 2016.

3.4. Sampling

Non- probability sampling – Quota water samples were selected randomly.

3.4.1. Sample size

Forty six known isolates of *E. coli* from drinking water have been isolated at Central Public Health Laboratory, Khartoum, Sudan.

3.5. Data collection

General data were collected from medical records of the samples based on a constructed questionnaire that included Date, place, type of water, etc... (Appendix III).

3.6. Sample processing

Forty six, *E. coli* isolates from drinking water were cultured in EMB media andstored at 4°C within three days.

3.7. Ethical consideration

Permission to carry out the study was obtained from the College of Graduate Studies, Sudan University of Science and Technology and Central Public Health Laboratory was informed for the purpose of the study before collection of the samples, a verbal consent was taken from them.

3.8. Laboratory works

The Gram stain was used to detect the Gram –negative bacilli, then cultured in EMB media, biochemical test was done to identify the *E. coli* bacterium, the susceptibility test was used to detect the sensitivity of antibiotic, The extraction

of DNA, PCR and gel electrophoresis was used to detect the virulence factors (*Stx*, *IpaH*, *aggR* and *eae*) in DEC strains

3.8.1 Collection of samples

Water samples were obtained from places suspected of having fecal contamination. Insuccessful isolation,someimportantfactorswere considered(Cheesbrough,1995):

-The inside and outside the tap was cleaned and disinfected carefully.

- The tap was opened and the water was flowed for 2-3 minutes

-The tap was turned off and the spout was sterilized by alcohol

-Water sample was taken with the sample container "bottles"

3.8.2Culturesof water samples:

The cultures was used for the detection of coliform bacteria in water using the presence-absence coliform test (Clark,1980).

The bottlecontent(50ml) of sterile selective culture broth containing lactose and an indicator"Laurytryptose(lactose) broth" (Appendix-11) with the Durham tube was added to an equal volume(50 ml) of water sample.

After incubation (48 hours at 37°C) the bottle in which lactose fermentation with acid and gas production has been cultured in two bottles of Birilent Green Bile Broth (BGBB) media (Appendix-II) with the Durham tube.

Two bottles were incubated in (BGBB) media for 48 hours in 37°C for one bottle to detect the coliform and the other one in 44°C to detect the heat-tolerant coliform.If a bottle in 37°Cshowed turbidity with acid andgas in the Durham tube in less than 48 hours this will be indicated for the presence of coliform bacteria.Then the bacteriawere identified by subcultcuringonEMB andIndole test.

3.8.3 Isolation of *E. coli*

3.8.3.1 Gram stain method

A primary stains "Crystal violete" was applied to the dry – heat – fixed smear of microorganism for 1 minute. Then the stain was washed with distilled

water and cover with Lugol's iodine for 1 minute. Stain was washed with distilled water.anddecolorized by eacetone, alcohol and washed with distilled water. Then the stain was coveredwithsafarnin for 2 minutes. The slide was placed in a rack to dry. The specimen was examined at (X100) (oil-immersion lens) (Cheesbrough, 1995).

3.8.3.2. Bacterial isolates

One loopful of the sample was placed in the top of the EMB agar plate and was streakeddown, the plate was then incubated overnight in 37°C in an incubator.A greenish metalic'sheen color was the indicator for a positive reaction of the presence of *E. coli* in water specimen (Cheesbrough, 1995).

3.8.3.3 Biochemical tests

Four biochemical tests were used for the identification of bacterial isolates,Indole test – Urease test – Citrate test – KIA test

A- Indole test method

Several colonies of the microorganism were rubbed into the tube of tryptophan peptone water media with sterile loop, the tube was incubated overnight in 37°C in an incubator, after 18-24 hours Kovacs reagent was added, a bright pink – red color ring was developed in positive reaction (Cheesbrough, 1995).

B - Urease test method

Colonies of the microorganism to be inoculated in the urea media by sterile loop, the tube was incubated overnight in 37°C , after incubation the media was tested for change in color if pink the test will be positive (Cheesbrough, 1995).

C- Citrate test method

Colonies of the microorganism were picked up by a straight wire and inoculated in sloped Simmon's citrate agar and incubated overnight at 37°C in an incubator, after incubation the media was tested for change in color if blue the test is positive (Cheesbrough, 1995).

D- KIA test method

Colonies of the microorganism were picked up by a straight wire and inoculated in sloped (KIA) media, then incubated overnight at 37°C in an incubator after incubation if there was achange throughout the medium, butt and slant are yellow, gas bubbles in the butt and no blackening in the butt this may be *E. coli* (Cheesbrough, 1995).

3.8.4 Susceptibility test

All the isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer disc diffusion technique (Bauer *et al.*, 1966), as per recommendation of CLSI, (2011).

The inoculums were prepared by using sterile loop, 4-5 colonies of similar appearance were takenand suspendedin 2 ml of sterile saline, the saline tube was vortexes to create smooth suspension. The suspension was compared with the turbidity standard of McFarland 0.5 (Appendix II) to adjust the density of the test suspension. Sterile cotton swab was immersed into the inoculums tube and the swab was rotated against the side of the tube suing firm pressure to remove excess fluid. The swab was streaked all over the surfaced dried Mueller – Hinton plate three times, finally, the swab was passed around the edge of the agar surface. Inoculums were left to dry for a few minutes at room temperature with the lid closed. A sterile forceps were used to place the anti-microbial discs (AppendixIII) uniformly and slightlypressured.The plates were then incubated in 35°C overnight. After overnight inculcation, the zone size of inhibition was measured and recorded in mm by using Ruler. Zone size was interpreted as per CLSI recommendation (CLSI. 2011). The results of susceptibility test were reported as susceptible, intermediate and resistant.

3.9.5 The DNA extraction

The DNA was extracted byboiling centrifugation method as described by Sherfi, (2007).

3.9.5.1Boiling centrifugation method

Several colonies of the isolatedorganism (*E. coli*)weresub cultured in Nutrient Agar media, afterovernight incubation at 37°C, 1-3 colonies were washed with 1ml sterile Normal Saline (NS) in sterile 1.5 ml Eppendorf tube. The tube was vortexed at 10.000r/min for 1-2 minutes. The supernatant was discarded.The pellet was resuspended in 200/μL, of distilled water.Thenthe tube was boiled at 95°C for 15 minutes.The tube was centrifuged at 13.000r/min for 3 minutes. The supernatant (that content the DNA) was removed to a new sterile Eppendorf tube. The tube was stored at ˉ 20°C till used.

3.9.5.2.Measurement of DNA concentration

Concentration of extracted DNA was read using gel electrophoresis to show the presence and quality of DNA in the sample,whencompared with a DNA marker of known concentration.

3.9.6Multiplex Polymerase Chain Reaction (MPCR)

Multiplex PCR was used for the detection of target genes; *IPaH*for EIEC, *AggR* for EAEC, *Stx*for EHEC and *eae*for EPEC.

1-Primers

PCR amplification was performed using published primer pairs (Table 3-1).

Table3-1: Primers sequences used for detection of virulence genes in diarrheagenic*E.coli*.

Primer name		**Sequence 5-3**	**Gene**	**Product size**	**References**
IPaHI	F	GTTCCTTGACCGCCTTTCCGATACCGTC	*IPaH*	619 bp	Saad*et al.*, 2011
IPaH2	R	GCCGGTCAGCCACCCTCTGAGAGTAC			
AggRKs1	F	GTATACACAAAAGAAGGAAGC	*AggR*	254 bp	Ogata *et al*., 2001
AggRKas2	R	ACAGAATCGTCAGCATCAGC			
VTcom-u	F	GAGCGAAATTTAAATTATGTG	*Stx*	518 bp	Saad*et al.*, 2011
VTcom-d	R	TGATGATGGCAATTCAGTAT			
SK1	F	CCCGAATTCGGCACAAGCATAAGC	*eae*	881 bp	Phantouamath*et al.*, 2003
SK2	R	CCCGGATCCGTCTCGCCAGTATTCG			

2-Preparation of Primers

For 100pmol/μL, each primer was dissolved in D.W as follows; *AggR – F* in 220μL D.W, *AggR – R* in 250μLD.W, *Stx – F* in 210μLD.W, *Stx*, R in 220μLD.W, *IPaH-F* in 250μLD.W, *IPaH-R* in 240μLD.W, *eae – F* in 250μLD.W, *eae-R* in 220μL D.W. And for 10 pmol/μL, 10μL of each primer was dissolved in 90μL of D.W.

3-Prparation of 10X TE buffer

Amount of 108 grams of Tris base were added to 55 grams of boric acid and 40ml of 0.5 EDTA, and then dissolved into 1liter of distilled water (pH8.0).

4-Preparation of IX TE buffer

10 ml of 10X was added to 90mL distilled water and heated until completely dissolved.

5-Preparation of ethidium bromide (10mg/ml)

Five milligrams of ethidium bromide powder were dissolved in 50ml DW and kept in brown bottle.

6-Preparation of agarose gel (2%)

Amount of 2.0g of agarose powder were dissolved in 100mL TE buffer. Then the mixture was heated in microwave for 90seconds and cooled to 60°C in room temperature. Then 3μL of (10mg/ml) ethidium bromide were added. Mixed well and poured into a casting tray with a comb. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification of the gel, the comb was gently removed.

7-Master Mix

Master mix (iNtRoN Biotechnology, Korea) was a remixed ready used solution containing all reagents required for PCR (except water, template DNA and primers) and additional compound needed for direct loading onto an agarose gel.

8-Preparation of reaction Mixture

The following reagents were used for each reaction in the following volumes (totalreaction volume was 25μL) in 0.2 ml eppendroff tube.

1- 17μL distilled water
2- 5μL master mix (iNtRoN Biotechnology, Korea).
3- 0.5μL forward primer (Macrogen Company, Korea).
4- 0.5μL reverses primer (Macrogen Company, Korea).
5- 2.0 μL DNA (Template DNA)
6- **Protocol used for amplification of the four genes (*AggR, IPaH, Stx* and *eae*).** The amplification was done by using Techne Tc.312Thermal cycle (UK). The PCR mixture was subjected to initialdenaturation step at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and the final extension at 72°C for 5 minutes.
7- **Visualization of PCR products**

The gel casting tray was flooded by 10X TBE buffer near the gel cover surface, then 5μL of amplified PCR products of each sample was put into each well. Then to the first well of casting tray 3 μL of DNA ladder (100bp) was injected for each run. The gel electrophoresis apparatus was connected to the power supply (primer, 125V, 500 MA, UK). The electrophoresis were done at 100V/cm for 30min, after that, the gel was removed by gel holder and visualized by U.V. Transilluminater (Uvite-UK), the gel results were photographed using the Polaroid film.

8- **Data analysis**

After the samples were collected and processed, the data was recorded and analyzed. Statistical analysiswas carried out by using SPSS software, version 16, chi-square tests was used to check the statistical significance..The results were then presented in a form of tables and figures.

Chapter Four

Results

4- Results

4.1The prevalence of *E. coli* isolates according to a source of water

The results showed that there was a high frequency of *E. coli* isolatesintap water 40/46 (86.9%) and low frequency of isolates in Tank 1/46 (2.2%) as showed in figure 4-1.

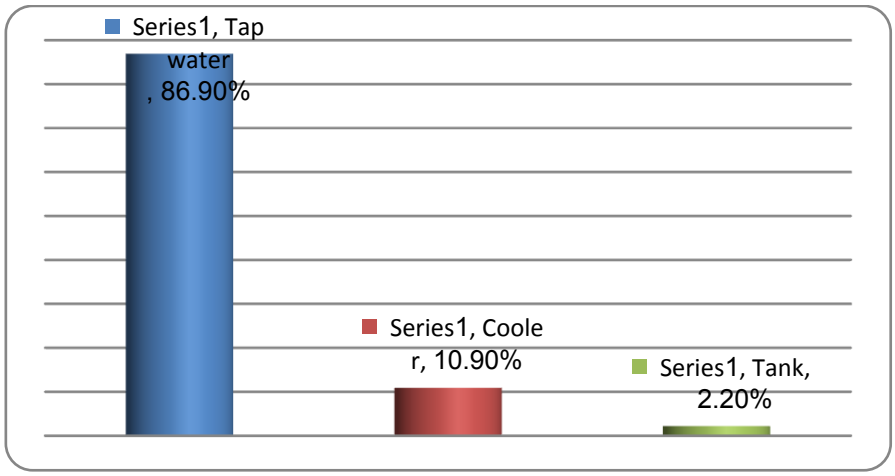


Figure 4-1: The prevalence of *E. coli* isolates according to a source of water.

4.2 The prevalence of *E. coli* isolates according to the place

The results showed that there was a high frequency of isolates from houses 36/46 (78.3%) followed by dormitory (15.2%) and (2.2%) from pharmacy, company and cafeteria in figure 4-2.

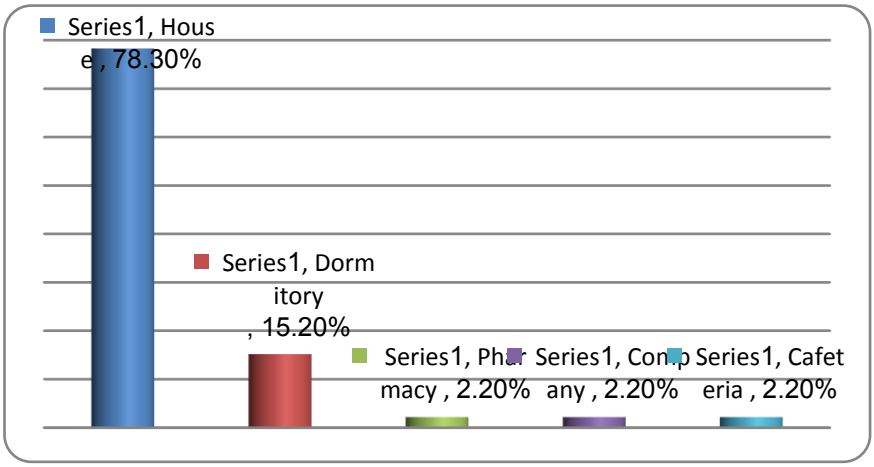
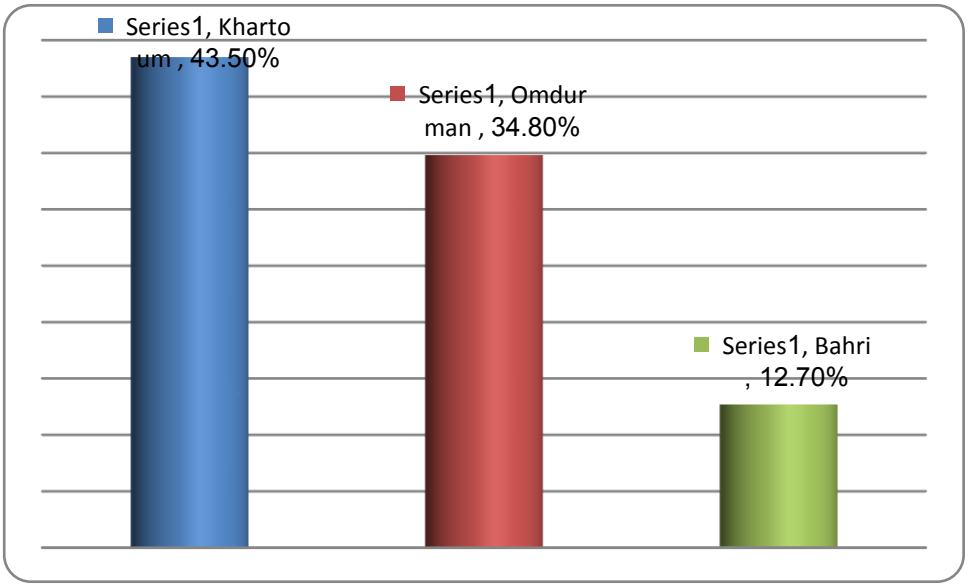


Figure 4-2: The prevalence of *E. coli* isolates according to the place.

4.3. The prevalence of *E. coli* isolates according to the ward and the province

The results showed that therewas a high frequency of *E. coli* isolates in drinking water in Khartoum as a province 20/46 (43.5%) as showed in figure 4-3.



4-3Figure: The prevalence of *E. coli* isolates according to the province.

4.4 Antimicrobial susceptibility of diarrheagenic*E. coli* isolated from drinking water samples

Antimicrobial susceptibility patterns of diarrheagenic*E. coli* isolated from drinking water, 67.4% of them were sensitive to Chloramphenicol, (63%) to Ceftriaxone, (52.2%) to Ciprofloxacin and (41.3%) to Gentamicin with high resistance rate to Tetracycline 97.8%,as shown in table 4-1

Table 4-1: The activity of antibiotics on *E. coli* isolated from drinking water.

Antibiotics	Sensitive	Resistance	Intermediate	Total
Chloramphenicol	31 (67.4%)	8 (17.4%)	7 (15.2%)	46 (100%)
Ceftriaxone	29 (63.0%)	4 (8.7%)	13 (28.3%)	46 (100%)
Ciprofloxacin	24 (52.2%)	11 (23.9%)	11 (23.9%)	46 (100%)
Gentamicin	19 (41.3%)	11 (23.9%)	16 (34.8%)	46 (100%)
Tetracycline	1 (2.2%)	45 (97.8%)	0 (0.0%)	46 (100%)

4.5 detection of diarrheagenic*E. coli* virulence genes isolate according to source of water and place.

The diarrheagenic*E. coli* virulence genes distribution according to the a source of water and place is shown the high frequency of virulence genes is IPaH 15 (32.6%) in tapwater, a high frequency of IPaH 19 (30.4%) in houses,as showed in table 4-2 and 4-3.

Table 4-2: The presence of virulence genes from *E. coli* isolates according to a source of water.

Source	*AggR*	*Stx*	*IPaH*	*eae*	*Total*
	+	+	+	+	
Tap water	11 (23.9%)	5 (10.8%)	15 (23.6%)	0 (0.0%)	31 (67.3%)
Coolerr	3 (6.5%)	0 (0.0%)	4 (8.7%)	0 (0.0%)	7 (15.2%)
Tank	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	14 (30.4%)	5 (10.09%)	19 (41.3%)	46 (100.0%)	38 (82.6%)

Key: + positive

Table 4-3: The presence of virulence genes of*E. coli* isolates according to the place.

Source	*AggR*	*Stx*	*IPaH*	*eae*
House	9 (19.6%)	4 (18.7%)	14 (30.4%)	0 (0.00%)
Dormitory	3 (6.5%)	0 (0.00%)	5 (10.8%)	0 (0.00%)
Pharmacy	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Company	1 (2.2%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Cafeteria	1 (2.2%)	1 (2.2%)	0 (0.00%)	0 (0.00%)

4-6 detection of diarrheagenic*E. coli* virulence genesisolates according to province.

The diarrheagenic*E. coli*virulence genes are distributed according to the province, there is a high frequency of *IPaH* gene 9 (19.6%) as shown in table 4-3

Table 4-4: The presence of virulence genes of*E. coli* isolates according to the province.

Source	*AggR*	*Stx*	*IPaH*	*eae*	*total*
	+	+	+	+	
Khartoum	6 (13.0%)	3 (6.5%)	9 (19.6%)	0 (0.00%)	18 (39.1%)
Omdurman	5 (10.9%)	0 (0.00%)	7 (15.2%)	0 (0.00%)	12 (26.0%)
Bahri	3 (6.5%)	2 (4.3%)	3 (6.5%)	0 (0.00%)	8 (17.3%)
Total	14 (30.4%)	5 (10.9%)	19 (41.3%)	9- (0.00%)	38 (82.6%)

Key: + positive

4-7Detection of virulence factors of *E. coli* isolates in the drinking water

Out of 46 isolates, 27 (58.6%) were positive for *IPaH, Stx* and *AggR* genes while 19 (41.4%) were negative for all these genes.*IPaH* gene was detected in 10 (12.7%), *AggR* gene was detected in 3 (6.5%) and Stx gene was detected in 3 (6.5%). Both *AggR* and *Stx* were co-existed in 9 (19.5%), both *AggR* and *IPaH* were co-existed in 2 (4.3%) and there was no *eae* gene detected in all isolates 46(100.0%),as shown in table 4-5 and 4-6.

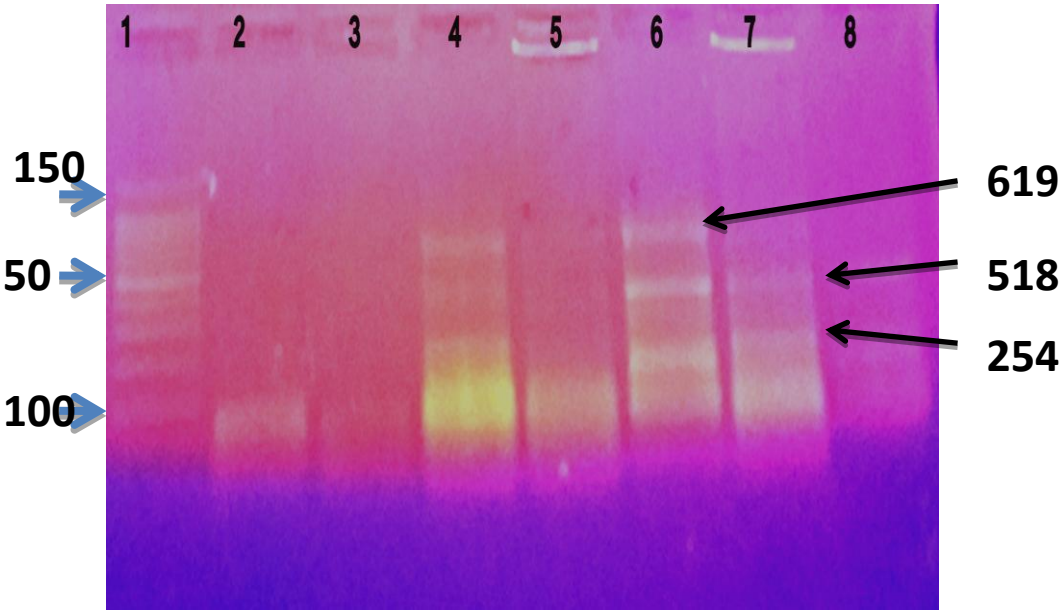


Figure 4.4: Multiplex PCR of *E.coli* strains using virulent specific primers. Lane 1: Gene ruler100bp; lane 2: negative control. Lane 3: Negative sample. Lane 4, 5, 6, 7 and 8 Multiplex of *eae* (EPEC), *ipaH* (EIEC), *stx* (EHEC), *AggR* (EAEC); *ipaH* (619bp); *stx* (518bp) and *AggR*(254bp).

Table 4-5: The prevalence of virulence gene among *E. coli* strains isolated from drinking water.

Sample	Genes				All genes
	IPaH	*AggR*	*Stx*	*eae*	
Positive	10 (21.79%)	3 (6.5%)	3 (6.5%)	0 (0.0%)	0 (0.0%)
Negative	36 (78.3%)	43 (93.5%)	43 (93.5%)	46 (100.0%)	19 (41.3%)
Total	46 (100.0%)	46 (100.0%)	46 (100.0%)	46 (100.0%)	19 (41.3%)

Table 4-6: Prevalence of co-existence of virulence genes among *E. coli* strains isolated in drinking water.

Valid	Frequency	Percent
AggR and *IPaH*	9	19.5%
AggR and *STX*	2	4.3%
Total	46	100.0%

Chapter Five

Discussion

5. Discussion

5.1. Discussion

This study was focused on the molecular detection of virulence genes of diarrheagenic *E.coli* in drinking water in Khartoum State, Sudan. A total of 46*E. coli* isolates were collected from drinking water from different regions in Khartoum State.

In the present study, we found a high prevalence of EIEC (*IPaH*gene) in Khartoum State drinking water. Most of them from tap water (32.6) in houses (30.4%), this finding is in agreement with a study in Japan by Yatsuyanagi *et al.* (2013), whom reported that EPEC wasthe most common strain in tap water. But itdisagredwith Dobrowksy*et al.,* (2014),in South Africa, theyreported that the most common source of water was tank water by EAEC.

This may indicate that the tap water was provided from its resource without chlorination or inefficiency chlorination.

In the present study it was found that the prevalence of diarrheagenie*E. coli*are more in Khartoum province (43.5%) and low in Bahri province (21.7%), this may be due to that Khartoum province, is bigger than Bahri province and consist of many wards, and the Khartoum has an old sanitation system with multi brokenpipes-line.

The antimicrobial susceptibility profile of *E.coli* is showed high rates of antibiotic resistance for Tetracycline (97.8%) similar findings for this study have been reported in South Africa by Adefisoye and Okoh, (2016). This may be due to long-term usage of Tetracycline (introduced in 1948); However *E.coli*was highly susceptible to Chloramphenicol (67.4%) Ceftriaxone (63%), Ciprofloxacin (52.2%) and Gentamicin (41.3%). This finding in the present study was more different from that obtained in other studies, Allocati*et al.,* (2013) whofound that antimicrobial resistance is on the rise in European countries to Ciprofloxacin, Gentamicin, and Ceftriaxone. In Chad,Adugna*elal.*, (2015) reported that *E. coli* was resistant to all antibiotic above.

Indicating that the antibiotic resistance by *E.coli* strains has increased in recent years. This could mainly be due to high consumption of antibiotic, irrational use, incomplete course of therapy, use of treatment without prescription from physicians.

The prevalence of virulence genes*(IPaH,AggR,Stx*and*eae)* associated withEIEC, EAEC EHEC and EPEC, respectively was done using PCR, was found that the most prevalent gene detected during this study was *IPaH*(EIEC) (N=10/46)(21.7%), followed by *AggR*(EAEC*)* and *Stx (*EHEC) (N=3/46) (6.5%) isolated for each. This finding in the present study is in disagreement with Roman *et al.,* (2013) in Mexico, whom found that the pathogenic type that was most commonly isolated was *EPEC* followed by EAEC. In Sidhu*et al.,* (2013),study from Australia whom found that theEHEC and EPECpathotype were the most prevalent genetically, in Gutierrez *et al.,* (2014) whom found that the ETEC was the most common in Mexico.

Indicating that, may the water purification system, hygiene practices and the source of water which can be differ from region to region and from country tocountry.

In contrast to genes results from 46 isolates tow combination of *E.coli* Isolates from one sample, *AggR(* EAEC*), Stx*(EHEC)(N=2/46) (4.3%). This finding in the present study was in agreement with those reported by Sidhu*et al.,* (2013) in Australia whom found that 4% of isolates were observed to have a combination of gene of both *EHEC* and *EAEC* path types, and in Ngugen*et al.*,(2016) in Vietnum whom found one sample (0.5%) contained the *Stx1*(EHEC) and *AggR* (EAEC). This finding may reflect recent fresh fecal contamination of surface water with diarrheagnic*E. coli.*

Other combination of *E.coli* was isolated from one sample *AggR* (EAEC), *IPaH*(EIEC), (N=9/46)(19.5%). This finding in the present study is not in agreement with any other studies.

The detection of Intimin*(eae)* gene associated with *EPEC* strain was not found in any drinking water sample collected during the sampling periods.

The most important feature of diarrheal disease due to EPEC infection is remarkable age distribution. EPEC infection is primarly a disease of infants younger than one year of age (Nataro And Karper, 1998). In the present study the isolate was from drinking water.

The prevalence of diarrheagenic *E.coli* strains in drinking water in general population of African countries have been reported to be (48%) of all diarrheagnic*E.col*i is EAEC in Libya (Ali *et al.,* 2011) and to be (35.3%) of all diarrheagenic *E.coli* is EPEC in Egypt (Hassanain*et al.,* 2015) and to be (34.7%) of all diarrheagenic *E.coli* is EPEC in Chad (Bessimbaye*et al.*, 2013). Which was differing from the present study findings.This might be due to important regional difference in the prevalence of categories of DEC.

5-2 Conclusions:

There is a high prevalence of Diarrheagenic strains of *E.coli* in drinking water in Khartoum State.The water can also be a source of transmission of drug resistant isolates. There is a high prevalence of EIEC (*IPaH*gen) in drinking water in Khartoum State. Detection of Diarrheagenic*E. coli* by Multiplex PCR was quite satisfactory.There is a high percentage of DEC in tap water; this may indicate that the contamination of drinking came from the source.

5-3 Recommendations:

1 -Khartoum State drinking water must be treated before supplied to human.

2- The drinking water should be periodically screened for the presence of bacterial contaminatesto prevent serious health risk

3- Large sample size is critical for best result.

4- Multiplex PCR technique should be used beside traditional laboratory methods as a routine technique in the diagnosis of Diarrheagenic*E. coli* strains in drinking water.

5- 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.

6- The houses should use filters in taps' spouts to filter the water before use.

7- The presence of antimicrobial resistance genes must be confirmed by more studies.

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Appendix I

Color plates

Plate 1: The Microwave

Plate 2: transilluminator

Plate 3: thermocycler

Appendix II

1:Crystal violet (HilMedia Laboratories Pvt. Ltd. Mumbai, Inida).

Ingredients	g/L
Crystal violet	20g
Ammonium oxalate	9 g
Ethanol or methanol, absolute	95 ml.

Preparation:

Weigh the crystal violet on a piece of clean paper (pre weighed), transfer to a brown bottle, pre marked to hold I liter, add the absolute ethanol or methanol (technical grade is suitable) and mix until the dye is completely dissolved, weight the ammonium oxalate and dissolve in about 200 ml of distilled water, add to the stain, make up to the I liter mark with distilled water, and mix well (Caution: Ammonium oxalate is a toxic chemical, therefore handle it with care), label the bottle, and store it at room temperature. The stain is stable for several months.

2: Lugol's iodine (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Potassium iodide	20g
Iodine	10 g.

Preparation:

Weight the Potassium iodide, and transfer to a brown bottle pre marked to hold I liter, add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved, weight the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved (Caution: iodine is injurious to health if inhaled or allowed to come in contract with eyes., therefore handle it with care in a well ventilated room and make up to the I liter mark with distilled water, and mix well. Label the bottle, and mark it Toxic. Store it in a dark place at room temperature Renew the solution if it'scolour fades.

3: Acetone-alcohol decolorize (HiMedia Laboratories Pvt. Ltd. Mumbai , India).

Ingredients	g/L
Acetone	500Ml
Ethanol or methanol, absolute	475 Ml

Preparation:

Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methy1 alcohol), transfer the solution to a screw-cap bottle of 1 liter capacity, technical grade is adequate, measure the acetone, and add immediately to the alcohol solution mix well (Caution: Acetone is a highly flammable chemical that vaporizes rapidly, therefore use it well away from an open flame) and label the bottle, and mark it Highly flammable. Store in a safe place at room temperature the reagent is stable indefinitely.

4:Safranin (HiMedial Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Safranin O	0.50
Ethyl alcohol, 95%	100.11

5: Eosin Methylene Blue "HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptone	10.000
Lactose	10.000
Dipostassuim Hydrogen phosphate	3.000
Eosin yellow dye	0.400
Methylene Blue dye	0.065
Agar	14.000

Directions:

Suspend 37.5 g in 1000 ml of cold distilled water. Heat to boiling, string constantly, distribute and autoclave at 121 $^{\circ}$C for 15 min cool to about 60°C and before transferring to plates gently shake the flask to oxidize the medium and

to disperse the flocculent precipitate that forms darning sterilization final PH 7.1 +/-0.2.

6: 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 1000 ml 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.

7:Kligler Iron Agar (HiMedia Laboratories Pvt.

Ingredients	g/L
Peptic digest of animal tissue	15.00
Beef extract	3.00
Yeast extract	3.00
Protease peptone	5.00
Lactose	10.00
Dextrose	1.00
Ferrous sulphate	0.20
Sodium chloride	5.00
Sodium trisulphate	0.30
Phenol red	0.024
Agar	15.00

Final pH 7.4 ±0.2(at 25°C).

Preparation

Suspend 57.52 grams in 1000 ml distilled water. Heat to boil to dissolve the mediumcompletely. Mix well and distribute into tubes. Sterilize by autoclaving

at 15 lbs pressure (121° C) for 15 minutes. Cool the tubeson slopes with 1 inch butts.

8: Peptone Water (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredients	g/L
Peptic digest of animal tissue	10.00
Sodium chloride	5.00

FinalpH7.2+/- 0.2(at 25°C).

Preparation

Suspend 15.0 grams in 100 ml distilled water. Mix well and dispense into tubes with or without inverted Durham's tubes and sterilize by autoclaving at15 lbs pressure (121°C) for 15 minutes.

9: Kovac's Reagent (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
P-dimethylaminobenzaldehyde	10 g
isoamyl alcohol	150mL
Concentrated hydrochloric acid	50mL

Preparation

Kovac's reagent is prepared by dissolving 10 gm of p-dimethyl aminobenzaldehyde in 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid.

10: Urea Agar (Christensen) (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredients	g/L
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Dipotassiumphosphate	1.20
Monopotasium phosphate	0.80
Phenol red	0.012
Agar	15.00

Final pH 7.4±0.2(at25°C).

Preparation

Suspend 21 grams in 950 ml distilled water. Heat to boiling todissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121° C) for 15 minutes.

Cool to 50°C and aseptically add 50 ml of sterile 40% urea solution and mix well. Dispense into sterile tubes and allow setting on slanting position. Don't over heat or reheat the medium as urea decomposes very easily.

11: Simmons Citrate Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymole blue	0.08
Agar	15.00

Final pH 7.4±0.2(at25°C).

11.Preparation

Suspend 24.28 grams in 100 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired in tubes or flasks, sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

12: Muller Hinton agar

Muller Hinton agar is used for testing susceptibility of common and rabidly growing bacteria using antimicrobial disc, it manufactured to contain low level of thymine, thymidine, calcium and magnesium.

Compositions

Ingredients	**g/L**
Casein acid hydrolysate	17
Beef heart infusion	2
Starch soluble	1.5
Agar	17
Final pH 7.2 +/- 0.2(at 25°C)	7.3

Directions

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

13: McFarland's standard (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredient	g/1
Sulphuric acid	1.0 ml
Barium chloride	2.35g
Distilled water	299 ml

Preparation:

Prepare 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of distilled water, prepare 1.175% w/v solution of barium chloride in 200 ml of distilled water, to make turbidity standard add 0.5ml of barium chloride to 99.5 ml of sulphuric acid and mix.

14:Lauryltryptosebroth(HiMedia Laboratories Pvt Ltd. Mumbai,India)

Ingredients	g/L
Tryptose	20.0 g
Lactose	5.0g
Dipotassiumhydrdgen phosphate K2HPO4	2.75g
Potassium dihydrogen phosphate KH2PO4	2.75g
Sodium chloride Nacl	5.og
Sodium Lauryl sulfate	0.1g
Reagent grad water	1L

PH7.4+0.2(at 25°C)

Preparation

Suspend 35.6 grams of the medium in one liter of distilled water,mix well and dissolve by heating with frequent agitation .Dispense into tubes with Durham gas collecting tubes for gas detection .Sterilize in autoclave at 121c for 15 minutes .Cool as quickly as possible.The prepared medium should be stored at 2-8°C.

15:BirilentGreenBileBroth (**BGBB**) (HiMedia Laboratories PvtLtd.Mumbai. India2)

Ingredients	gL
Tryptone	10.0g
Bacteriological ox bile	20.0g
Lactose	10.0g
Brilliant green	13.3mg
Distilled water	1L

PH 7.2+ 0.2 (at 25°C)

Preparation

Dissolve 4o.0 grams of dehydrated medium in 1 liter of distilled water .Stir slowly until complete dissolution .Dispend in appropriately-sized tubes containing a Durham tube .Sterilize in an autoclave at 121c for 15 minutes .After cooling,theDurham tubes should not contain trapped air.

Appendix III

Questionnaire

Detection of virulence factors of diarrheagenic E.coli strain (EIEC, EHEC, EPEC and EAEC) in drinking water isolates – Khartoum, Sudan.

By:

OmimaAbdEljalil

Date: ……..

No:……

Type of water:……

Place: ……

Province: …….

1: Antibiotic disc

Gentamicin	GEN	10 mg/disc
Ciprofloxacin	CIP	5 mg/disc
Ceftriaxone	CTR	30 mg/disc
Chloramphenicol	C	30mg/disc
Tetracycline	TE	30mg/disc

2: Data of Diarrheagenic*E. coli* isolated in drinking water in Khartoum State

	AggR	Stx	IPaH	eae	CIP	C	CTR	GEN	TE	Source	Place	Province
1-	Negative	Negative	Negative	Negative	ensitive	Sensitive	Sensitive	Sensitive	Resistant	Tap	House	Khartoum
2-	Negative	Positive	Negative	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tap	House	Khartoum
3-	Negative	Positive	Negative	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tap	House	Khartoum
4-	Negative	Negative	Negative	Negative	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Tap	House	Khartoum
5-	Negative	Negative	Negative	Negative	Intermediate	Sensitive	Intermediate	Intermediate	Resistant	Tap	House	Khartoum
6-	Positive	Negative	Positive	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tank	House	Khartoum
7-	Negative	Negative	Positive	Negative	Intermediate	Resistant	Resistant	Intermediate	Resistant	Tank	House	Khartoum
8-	Negative	Negative	Positive	Negative	Resistant	Sensitive	Intermediate	Intermediate	Resistant	Tank	House	Khartoum
9-	Negative	Negative	Negative	Negative	Resistant	Sensitive	Sensitive	Intermediate	Resistant	Tank	House	Khartoum
10-	Negative	Negative	Negative	Negative	Sensitive	Sensitive	Intermediate	Sensitive	Resistant	Tank	House	Khartoum
11-	Negative	Negative	Negative	Negative	Intermediate	Sensitive	Sensitive	Intermediate	Resistant	Tank	House	Khartoum
12-	Negative	Negative	Positive	Negative	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Tank	House	Khartoum
13-	Positive	Negative	Positive	Negative	Intermediate	Resistant	Intermediate	Resistant	Resistant	Cooler	Dormitory	Khartoum
14-	Positive	Negative	Positive	Negative	Resistant	Resistant	Intermediate	Resistant	Resistant	Cooler	Dormitory	Khartoum
15-	Positive	Negative	Negative	Negative	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Tank	Campany	Khartoum
16-	Positive	Positive	Negative	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Tank	Cafeteria	Khartoum

17- Negative	Negative	Positive	Negative	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Cooler	Dormitory	Omdurman
18- Negative	Negative	Positive	Negative	Intermediate	Sensitive	Intermediate	Intermediate	Resistant	Tank	Dormitory	Omdurman
19- Positive	Negative	Positive	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Cooler	Dormitory	Bahri
20- Negative	Negative	Negative	Negative	Resistant	Resistant	Sensitive	Resistant	Resistant	Tap	Pharmacy	Bahri
21- Negative	Negative	Negative	Negative	Resistant	Intermediate	Intermediate	Resistant	Resistant	Tap	House	Omdurman
22- Negative	Negative	Positive	Negative	Resistant	Intermediate	Intermediate	Resistant	Resistant	Tap	House	Omdurman
23- Positive	Negative	Negative	Negative	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Tap	House	Omdurman
24- Negative	Negative	Negative	Negative	Intermediate	Intermediate	Resistant	Resistant	Resistant	Tap	House	Omdurman
25- Negative	Negative	Positive	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tank	House	Omdurman
26- Positive	Negative	Positive	Negative	Sensitive	Sensitive	Resistant	Resistant	Resistant	Tank	House	Omdurman
27- Positive	Negative	Positive	Negative	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Tank	House	Omdurman
28- Negative	Negative	Positive	Negative	Resistant	Intermediate	Intermediate	Resistant	Resistant	Tank	House	Omdurman
29- Negative	Negative	Positive	Negative	Intermediate	Resistant	Intermediate	Intermediate	Resistant	Tank	House	Omdurman
30- Positive	Negative	Negative	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tank	House	Omdurman
31- Negative	Negative	Negative	Negative	Intermediate	Sensitive	Sensitive	Intermediate	Resistant	Tank	House	Omdurman
32- Positive	Negative	Positive	Negative	Sensitive	Resistant	Sensitive	Sensitive	Resistant	Tank	1House	Omdurman
33- Negative	Negative	Negative	Negative	Sensitive	Resistant	Sensitive	Sensitive	Resistant	Tank	House	Omdurman
34- Negative	Negative	Negative	Negative	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Tank	House	Omdurman
35- Negative	Negative	Negative	Negative	Sensitive	Resistant	Sensitive	Resistant	Resistant	Cooler	Dormitory	Bahri

36- Negative	**Negative**	**Negative**	**Negative**	**Intermediate**	**Intermediate**	**Sensitive**	**Intermediate**	**Resistant**	**Tank**	**Dormitory**	**Bahri**
37- Negative	**Negative**	**Positive**	**Negative**	**Sensitive**	**Sensitive**	**Intermediate**	**Intermediate**	**Resistant**	**Tank**	**House**	**Khartoum**
38- Positive	**Negative**	**Positive**	**Negative**	**Intermediate**	**Intermediate**	**Sensitive**	**Resistant**	**Resistant**	**Tap**	**House**	**Bahri**
39- Negative	**Negative**	**Negative**	**Negative**	**Intermediate**	**Sensitive**	**Intermediate**	**Sensitive**	**Resistant**	**Tap**	**House**	**Bahri**
40- Negative	**Negative**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Sensitive**	**Intermediate**	**Resistant**	**Tank**	**House**	**Bahri**
41- Negative	**Negative**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Sensitive**	**Intermediate**	**Resistant**	**Tank**	**House**	**Bahri**
42- Negative	**Positive**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Tank**	**House**	**Bahri**
43- Positive	**Negative**	**Positive**	**Negaive**	**Resistant**	**Intermediate**	**Intermediate**	**Resistant**	**Resistant**	**Tank**	**House**	**Bahri**
44- Negative	**Negative**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Tank**	**House**	**Bahri**
45- Positive	**Positive**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Sensitive**	**Intermediate**	**Resistant**	**Tank**	**House**	**Bahri**
46- Negative	**Negative**	**Negative**	**Negative**	**Sensitive**	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Tank**	**House**	**Bahri**