Bacterial Causes of Diarrhea Among Some Sudanese Patients – Khartoum State, Sudan

By:

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A Thesis
Submitted in the Fulfillment of the Requirements for the Degree of Doctor of Philosophy
In
Microbiology

Faculty of Post-graduate Studies, University of Gezira

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Date: July, 2013
Dedication

To my mother
To the soul of my father
&
To my family
Acknowledgement

Grateful thanks are due to my supervisors Prof. Samia Ahmed Gumaa and Prof. Ahmed Abddalla Mohamadani for their guidance, advice, follow up and supervision.

Cordial gratitude is due to my colleague, and sincere friend Omer Balla for his absolute, endless support and encouragement throughout my study.

Special thanks to Major General Eiz Eldien Mahmoud, chairman of the Medical Laboratories, Omdurman Military Hospital for his valuable help while collection of samples.

My thanks are extended to the Microbiology staff, Faculty of Medical Laboratory Sciences U. of K, especially for Samira and Mona for their help through my practical work.

I owe special thanks and gratitude to Dr. Omer Osman for analyzing the data.

Thanks go to Dr. Abddalla Osman, Miss. Nada A/Ghani and Mrs. Omnia Ibrahim for their extreme help and effort all throughout my study.

Last but not least my acknowledgement is extended to my family for their patience and moral support through my study.
Bacterial Causes of Diarrhea Among some Sudanese patients – Khartoum State (2009-2013)

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Abstract

Diarrheal disease remains a major public health problem throughout the world, with over more than 3 million deaths occurring each year, mostly among children. Many types of bacteria can act as causative agents of diarrhea. Some of these agents are not reported in Sudan, and their antimicrobial susceptibility pattern are not well known. The aim of this study is to determine the bacterial causes of diarrhea among Sudanese patients and their antimicrobial susceptibility pattern in Khartoum State. Stool specimens were collected from 900 patients with diarrhea, during the period of Jan. 2009 to Feb. 2012. All specimens were cultured using different types of selective media which included: DCA, XLD, TCBS, HE, MacConkey No.3 and Skirrow media. The suspected organisms were identified by conventional biochemical tests, API 20E system and serological tests. Two hundred thirty-two (25.77%) isolates were identified as enteric pathogens. The most frequently isolates were diarrheagenic E. coli strains 124 (53.45%) followed by Shigella species 38 (16.38%), Salmonella species 19 (8.19%), Campylobacter species 15 (6.47%), Aeromonas hydrophila 15 (6.47%), Morganella morgani 13 (5.6%), Yersinia enterocolitica 5 (2.16%) and non-cholera Vibrios 3 (1.29%). The results of the antimicrobial susceptibility test showed that 225 (96.98%) of all isolates were susceptible to ciprofloxacin, whereas 212 (91.38%) and 203 (87.5%) were susceptible to ceftriaxone and cefixime respectively. The susceptibility to gentamicin among all isolates was (73.28%) and to cotrimoxazole (39.34%). Only 4 (1.72%) were susceptible to amoxy-clavulanic acid. Ten strains of E.coli 0157 were reported in this study, although it causes serious type of diarrhea, it can be easily isolated and identified even in laboratories with low facilities. The pathogens, Aeromonas, Yersinia, Morganella and non-cholera Vibrios, isolated in this study, may be reported for the first time as causative agents of diarrhea among Sudanese patients in Khartoum State.
أنواع البكتيريا المسببة للاسهال بولاية الخرطوم
الأمين محمد إبراهيم خوجلي
دكتوراه الفلسفة في المختبرات الطبية (الأحياء الدقيقة 2013م)
قسم الأحياء الدقيقة
كلية علوم المختبرات الطبية
جامعة الجزيرة

ملخص الدراسة

تعتبر الاسهالات من أكبر المشكلات الصحية بالدول النامية حيث يسجل سنويا أكثر من 3 ملايين حالة وفاة خاصة بين الأطفال. أنواع كثيرة من البكتريا يمكن أن تسبب الاسهالات الجرثومية، ولكن كثير منها لم يتم عزله أو تصنيفه بالسودان كما لا توجد بيانات كافية عن نمط حساسية هذه البكتريا للمضادات الجرثومية.

الغرض من هذه الدراسة عزل وتوصيف البكتريا الهوائية المسببة للاسهال من عينات البراز ثم تحديد حساسية هذه البكتريا لبعض من المضادات الجرثومية كذلك تهدف هذه الدراسة لعزل وتوصيف أي من الجراثيم المسببة للاسهال والتي لم يتم عزلها أو توصيفها في أي دراسة سابقة بالسودان. تم جمع عدد 900 عينة براز من الأشخاص الذين يعانون من الأسهال خلال الفترة من يناير 2009 إلى فبراير 2012. جميع العينات تم تزريعها في مجموعة من الأوساط العازلة والتي شملت أوساط ديوكسي كولايت ستريت، إكس إل دي، أتش اي، ماكونكي رقم 3، تي سي بي إس ووسط سكايرو. تم توصيف الجراثيم المعزولة بواسطة الاختبارات الكيميائية الاعتيادية، طريقة الاختبارات الرقمية التصويرية التحليلية وكذلك الاختبارات المصلية. أفرزت هذه الدراسة عن عزل وتوصيف عدد 232 من الجراثيم الممرضة بنسبة (25.8%). أكثر الجراثيم التي تم عزلها كانت جراثيم الأشريكية القولونية الاسهالية بعدد 124 (نسبة 53.5%) تلتها جراثيم الشقيلا بعدد 38 (نسبة 16.4%) وجراثيم السالمونيلا بعدد 19 (نسبة 8.2%) وجراثيم السيفيات بعدد 15 (نسبة 6.5%) وجراثيم الهولاندية المائية أيضاً بعدد 15 (نسبة 6.5%) وجراثيم المرغانية المائية بعدد 13 (نسبة 5.6%) وجراثيم المشيدة المائية للاعماء بعدد 5 (نسبة 2.2%) وجراثيم الضاربة الزرقاء بعدد 3 (نسبة 1.2%). اختبارات الجراثيم المعزولة ضد المضادات الجرثومية المستعملة أظهرت أن نسبة (97%) منها حساس لعقار سيروفرولوكساسين بينما (91.4%) منها حساس لعقار سيبيرفلوكساسين و (87.5%) منها حساس لعقار سترابكسون و (85.5%) منها حساس لعقار سيفرولوكساسين و(73.3%) منها حساسة لعقار جنتاميسين و (39.3%) منها حساسية لعقار سيربرولوكساسين و (39.3%) منها حساسية لعقار سوبرفولوكساسين و (39.3%) منها حساسية لعقار سوبرفولوكساسين و (39.3%) منها حساسية لعقار سوبرفولوكساسين.

وقد تم من خلال هذه الدراسة عزل 10 سلالات من جراثيم الأشريكية القولونية المائية التي تسبب نوعا خطيرا من الأسهال علمًا بأن هذا الجرثوم يمكن عزله وتوصيفه بسهولة في أي من مختبرات الأحياء الدقيقة كما تم لأول مرة من خلال هذه الدراسة عزل وتوصيف جراثيم الهولاندية المائية، والبرسيمية الملحية للاعماء، المرغانية المائية، الضاربة الزرقاء، غير الهيضية كجراثيم مسببة للاسهال بولاية الخرطوم.
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List of Abbreviations

CLSI: Clinical and Laboratory Standards Institute.
DAEC: Diarrhea associated haemolytic E. coli
DCA: Deoxycholate citrate agar.
DEC: Diarrheagenic Escherichia coli strains.
EAgg EC: Enteroaggregative E. coli.
EHEC: Enterohaemorrhagic E. coli.
EIEC: Enteroinvasive E. coli.
EPEC: Enteropathogenic E. coli.
ESBLs: Extended Spectrum β. lactamases.
ETEC: Enterotoxigenic E.coli.
HE: Hektoen enteric agar.
SLTEC: Shiga-like toxin producing E.coli.
TCBS: Thiosulphate citrate sucrose bile salt agar.
VTEC: Verotoxigenic E.coli.
XLD: Xylose lysine deoxycholate agar.
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CHAPTER ONE
: Introduction and literature review

1-1. Introduction:
Infections of the gastrointestinal tract are among the most frequent diseases worldwide, exceeded only by respiratory tract infections. Most episodes are usually mild and self-limiting except in the very young, the elderly and immunocompromised patients. (1,2,3)
The most prominent clinical features of these infections are abdominal pain, fever, vomiting and diarrhea, of which diarrhea is the commonest feature. (4,5,6)

Infectious diarrhea is a major public health problem and remains one of the most important causes of morbidity and mortality among infants and children especially in developing countries. It has been estimated that in Africa, Asia and Latin America a child's chance of dying of diarrheal diseases before the age of 7 years can be as high as 50%. In developed countries the rate is much lower (2,7,8,9).

1-1-2. Diarrhea:
Diarrhea is defined as an intestinal disorder characterized by abnormal fluidity and frequency of faecal evacuations, at least three times within a 24-hour period. (5)

Bloody diarrhea refers to any diarrhea episode in which the loose or watery stools contain visible blood. Watery diarrhea is a term used to describe watery stool that does not contain RBCs but may contain mucus or other components. It is mainly caused by enterotoxin producing organisms like ETEC and Vibrios. (6)

Diarrhea caused by bacteria that invade the bowel mucosa like Shigella and EIEC usually causes inflammation and tissue damage, characterized by presence of numerous polymorphnuclear leucocytes. (5,6)

Dysentery is a term used to describe the syndrome of bloody diarrhea with urgency, small volume of stool, fever, abdominal cramps and rectal tenesmus. This condition is mainly caused by Shigella species.

About 80% of deaths due to diarrhea occur in the first two years of life. The main cause of death from acute diarrhea is dehydration, which results from the loss of fluid and electrolytes in diarrheal stool. (1)
1-1-2-1. Aetiology of diarrhea:
A wide range of bacteria, both aerobic and anaerobic can act as aetiological agents of infectious diarrhea. Anaerobic bacterial agents include *Clostridium difficile*, *Enterotoxigenic Bacteriodes fragilis* (ETBF) and some strains of *Clostridium perfringens*.\(^{(10,11,12,13,14)}\)
A wide range of recognized aerobic enteric pathogens can cause diarrhea, among which, diarrhaegenic *Escherichia coli* (DEC) and *Shigella* spp. are the most common. Other pathogens include *Campylobacter* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Morganella morgani* and *Vibrio cholerae* which play an important role in many different geographic areas.\(^{(15,16,17,18,19,20)}\)
Many of these aetiological agents are not diagnosed in Sudan because they cause mild and self-limiting disease the patient does not seek medical attention or because medical and laboratory facilities are unavailable.

1-1-2-2. Epidemiology of bacterial diarrhea:
Yousef et al conducted study in the year (2000) which determined the etiology of acute diarrhea in Jordanian children. From 265 stool specimens. Enteropathogens were detected in (66.4%) of patients with diarrhea. The study showed that Rotavirus was the most common pathogen isolated (32.5%), followed by EPEC (13.8%), EAggEC (10.2%) ETEC (5.7%), *Shigella* spp. (4.9%), *Salmonella* spp (4.5%), *Campylobacter jejuni* (1.5%), EIEC (1.5%) and *Yersinia enterocolitica* (0.4%).\(^{(21)}\)
Medeiros et al studied the acute diarrhea among children in Ribeirao Preto, Brazil in the year 2001. From 1836 stool specimens enteropathogens were isolated from 419 (22.89%). The pathogens indentified by standard methods were the following *Escherichia coli*, *Salmonella* spp, *Shigella* spp., *Campylobacter* spp., and *Yersinia* spp.\(^{(22)}\)
el-Sheikh et al in the year 2001 investigated the prevalence of viral, bacterial and parasitic enteropathogens among children with acute diarrhea in Jeddah, Saudi Arabia in 576 faecal samples. One or more enteropathogens were identified in (45.6%) of the stool specimens. Among the bacterial isolates, diarrhaegenic *E. coli* strains constituted
(13%) as follows: (3.8%) were EPEC with serotype 0111 being more common, (1.9%) EHEC followed by Salmonella spp. (3%) and Shigella spp. (9)

In Porto Velho, Rondonia, Orlandi et al (2006), studied 130 cases of infectious diarrhea. He reported that Rota virus was the most common pathogen (19.2%) followed by Shigella (7.6%), Salmonella spp. (6.9%), ETEC (3.1%), EPEC (2.3%) EIEC (0.8%) and Yersinia enterocolitica (0.8%).(23)

1-1-3. Common bacterial causes of diarrhea:
1-1-3-1. Escherichia coli:
E. coli is a member of the family Enterobacteriaceae which comprise a large group of Gram negative bacilli. It is a normal inhabitant of the gastrointestinal tract of all warm-blooded animals, but variants of this species are also among the important etiological agents of gastroenteritis and several extraintestinal diseases. Over 700 antigenic types of E.coli are recognized based on somatic (O), flagella (H) and capsular (K) antigens. (24,25)

E. coli strains that cause diarrheal diseases are known as diarrhaegenic Escherichia coli strains (DEC). There are at least 7 categories of recognized DEC strains differentiated according to their virulence properties, mechanisms of pathogenicity, clinical symptoms and serology. These categories include: (25,26,27,28)

1-1-3-1-1. Enterotoxigenic E.coli (ETEC):
An important cause of diarrhea among young children in developing countries and a major cause of traveller’s diarrhea in adults from industrialized countries. (29)

It causes diarrhea by producing heat-stable (ST) , heat-labile toxin (LT) or both. The heat labile toxin is closely related in structure and function to the cholera toxin (CT) expressed by Vibrio cholerae. This toxin can be detected by many methods, of which multiplex PCR is the best because it is highly sensitive and specific. (27,30,31,32)

Enterotoxigenic E.coli infections usually follows ingestion of contaminated water and food, producing watery diarrhea, nausea, abdominal cramps and low-grade fever. In infants ETEC sometimes
causes a disease known as “Cholera infantum”. Most common serogroups of ETEC involved in diarrheal diseases include: 06-08-015-020-025 and 027. (24,25,33)

1-1-3-1-2. Enteropathogenic *E.coli* (EPEC):
It is recognized as a leading cause of infantile diarrhea in developing countries. It has been associated with prolonged non-bloody diarrhea with large amount of mucus. The pathogenesis is unclear; it does not produce enterotoxin or shiga toxin and is not invasive. Sequela may include malnutrition, weight loss and growth retardation. It can be diagnosed by serology, PCR and culture in Hela cells. Most commonly involved serogroups include 055-086-0111-0119-0126 and 0127. (24,33,34)

1-1-3-1-3. Enteroinvasive *E.coli* (EIEC):
This causes bloody mucoid diarrhea by a pathogenic mechanism similar to that of Shigella species by invading cells of the colon. Enteroinvasive *E.coli* are a significant cause of morbidity and mortality in young children in developed countries. The classical method for identification of EIEC is the Sereny test which demonstrates the ability of the organism to cause keratoconjunctivitis in the eye of guinea pigs. Also it can be diagnosed by serology and cell line culture using Hela cells. Few serogroups of *E.coli* can be involved in this condition which includes: 0112-0124-0136-0143-0144 and 0152. (35,36,37)

1-1-3-1-4. Enterohaemorrhagic *E.coli* (EHEC):
Enterohaemorrhagic E.coli belong to a number of serogroups, but those of serogroup 0157 are most important in human disease. The organism produces a toxin which has cytotoxic activity on Vero and Hela cells and hence is known as Verotoxigenic *E.coli* (VTEC). This toxin is very similar biochemically to the shiga toxin produced by *Shigella dysenteriae* type 1 and threfore termed shiga-like toxin producing *E.coli* (SLTEC). (25,38)

Many outbreaks caused by EHEC have been reported from various parts of the world including North America, Western Europe, Australia and Asia between 1997-2001. The organism has low infectious dose and is easily transmitted through contaminated water and food such as meat and
unpasteurized milk. Also zoonotic transmission may occur. It is considered among the most common causes of food-borne diseases worldwide. (39,40,41)

Human infection with EHEC is associated with many symptoms ranging from bloody and non-bloody diarrhea, fever and vomiting through to haemorrhagic colitis and haemolytic uremic syndrome; characterized by acute renal failure in young patients. (25,42)

The organism can be easily isolated from stools using MacConkey agar and sorbitol MacConkey agar (SMAC). There are various modifications of SMAC agar that improve selectivity and differentiation. Cefixime tellurite CT-SMAC is a good modified selective and differential medium for isolation of EHEC from stools. PCR and other modern techniques are also used for diagnosis of EHEC but are more expensive than culture methods. (43,44)

1-1-3-1-5. Enteroaggregative *E.coli* (EAggEC):-
These are so named due to their adherence pattern to culture epithelial cells in the presence of aggregative adherence fimbriae. The organism is usually associated with acute or persistent diarrhea in developing countries, particularly in children leading to malnutrition and growth retardation. (45,46)

In the developed world, several recent studies reported many outbreaks of food-borne illness due to EAaggEC. Many studies suggested that EAaggEC are the predominant causes of *E.coli*-mediated diarrhea in developed countries. (47)

Enterohaemorrhagic E.coli has been associated with diarrhea in children in Chile, Mexico and Kenya and with bloody diarrhea in children in India. Also it has been isolated from children with diarrhea during an outbreak in Japan. The pathogenesis of EAaggEC is not well understood, but recent studies proposed that it causes secretory diarrhea and mucosal damage which may be due to enterotoxins and cytotoxins. (45,48)

1-1-3-1-6. Diarrhea-associated haemolytic *E.coli*:-
These are also known as cell-detaching *E.coli* and diffusely-adherent *E.coli* (DAEC) so named because their virulence is mediated by α-haemolysin and cytotoxin necrotizing factor 1, and because of their
diffuse adherence pattern to cultured epithelial cells. It causes mucoid watery diarrhea without blood or fecal leucocytes. It has significant association with diarrhea in children, 1 to 5 years of age but not associated with illness in infants.\(^{(24,33,49,50)}\)

1-1-3-1-7. Cytolethal distending toxin-producing *E.coli* (CDT-producing *E.coli*).

This is recent addition to the diarrheagenic classes of *E.coli*. It causes distension and eventual disintegration of cells of certain lines.\(^{(33,51,52,53)}\)

**1-1-3-1-8 The epidemiology of diarrheagenic *E.coli*:**

The aetiology of traveller’s diarrhea on Caribbean Island, Jamaica was studied by Paredes P. et al in the year 2000 among travelers to 5 hotels. From 332 stool specimens, 118 recognized enteropathogens were isolated as follows:

ETEC (26.2%), *Salmonella* spp. (4.2%) and *Shigella* spp. (4.2%).\(^{(54)}\)

EPEC (10.8%), ETEC (2.4%) EIEC (1.2%) EHEC (0.6%), EAggEC (10.3%), DHEC (7.9%) and CDT-producing *E.coli* (6.9%).\(^{(55)}\)

Islam et al (2006) determined the prevalence of shiga toxin-producing *E.coli* STEC among hospitalized patients with diarrhea in Dhaka, Bangladesh using culture and PCR methods. From 410 stool samples, STEC was isolated from 5 (1.2%) samples.\(^{(56)}\)

Anupkumar et al (2007) studied the role of *Escherichia coli* in acute diarrhea in tribal preschool children of Central India. From 244 stool specimens, they reported the following:

Classical bacterial enteropathogens were isolated from Twenty-six children: *Shigella* spp. from 14, *Vibrio cholerae* from 7 and *Salmonella* spp. from 5 samples. EAggEC was isolated from 64, EPEC from 27, ETEC from 10 samples. Rotavirus, *Giardia* and *Entamoeba histolytica* were seen in different samples.\(^{(57)}\)

Al-Gallas et al (2007) examined 271 stool specimens collected from children and adults in Tunisia. The most frequently isolated enteric pathogens were ETEC (32.3%) EAEC (11.3%) EIEC (11.3%) EHEC (10.4%) and *Salmonella* spp. (9.5%).\(^{(6)}\)

Joseph et al (2007) determined the presence of carriage of EAEC, EPEC and ETEC genes among 1399 food handlers working in tourist hotels in
Kenya. EAEC genes was harboured by 29 (2.1%), 11 (0.8%) the EPEC and 2 (0.1%) had the STEC. The Antibiotic susceptibility profiles of the isolates showed the following resistance rates: Ciprofloxacin (0%), cefotaxime (3.5%), amoxy-clavulanic acid (6%), gentamicin (6%) and co-trimoxazole (72.4%).

Eizo Takahashi et al (2008) in Myanmar investigated 217 E.coli isolates from children for the presence of virulence genes related to diarrhea by colony hybridization and PCR. Isolates of 47 out of 217 children (21.7%) possessed virulence genes characteristic of diarrheagenic E.coli, 30 were EAEC, 12 were EPEC and 5 were ETEC.

A similar study was conducted by Toni et al (2008) in Brazil. Out of 306 stool samples, STEC was isolated from six (1.96%) samples. The isolates were susceptible to co-trimoxazole, nalidixic acid, cephalothin and gentamicin. Resistant only to ampicillin.

A prospective study was carried out by Lilian in Brazil (2008) in stool specimens of children less than 2 years of age with and without diarrhea. The specimens were examined for the presence of diarrheagenic E.coli using colony blot hybridization with specific DNA probes designed to detect EPEC, ETEC, EIEC, EAEC and EHEC/STEC.

Diarrheagenic E.coli strains were detected as the sole pathogen in stools of 92 (30.3%) children; in 72 (33.0%) with diarrhea and 20 (23.2%) without diarrhea. DAEC was the most frequent pathotype and was found significantly more often in patients (18.3%) than in controls (8.1%). Atypical EPEC and EAEC isolates were isolated from both patients and controls (5.5% and 4.6%) respectively. ETEC was more frequently isolated from patients (3.2%) than controls (1.2%). Typical EPEC (0.9%) and EIEC (0.4%) isolates were detected only in children with diarrhea.

In Japan, Hinenoya et al (2009) examined the prevalence of CTEC among children having diarrhea using a PCR-RFLP assay. Out of 362 stool specimens, 30 STEC strains were isolated.

Vincent et al (2010) assessed the prevalence of E.coli 0157 in patients with diarrhea and surface waters from some selected sources in Zaria, Nigeria, evaluating the antibiotic susceptibility and plasmid profiles of
184 *E. coli* isolates, obtained from 228 water samples and 112 diarrheal stool specimens using standard methods. The detection rate of *E. coli* 0157 in surface waters was 2.2% and its prevalence in children with diarrhea was 5.4%. The most active antibiotics were gentamicin, chloromphenicol and fluoroquinolones. From the 184 *E. coli* isolates, 79 (42.9%) were resistant to 4 or more antibiotics. Multidrug resistant (MDR) was higher amongst aquatic isolates than in clinical isolates. *Mariana et al* (2010) investigated the role and clinical course of VTEC infection in children with acute diarrhea from Argentina, the country with the highest worldwide incidence of haemolytic ureamic syndrome (HUS). To accomplish this objective, 437 samples from children up to 6 years old with acute diarrhea were collected and processed using multiplex PCR. From the 437 samples 44 (10.1%) were found positive for VTEC virulence genes VT1, VT2, eae and ehxA. VTEC infected-patients presented different types of diarrhea (27.3% belonged to the non-bloody type). Several serotypes and virulence genotypes were found. Isolates belonged to the serotypes 0157:H7, 0145, 026:H11, 0121:H19, 0111:H2 and 0118:H2. Haemolytic ureamic syndrome developed in 16 (36.4%) patients positive for VTEC virulence genes. All the VTEC isolates produced a cytopathic effect in Vero Cell monolayers, confirming the ability to express Vero toxin. *In Burkina Faso, Bonkaungou et al* (2011) investigated the occurrence of five major DEC pathogroups in stool samples from 658 children with diarrhea. DEC strains were isolated from 214 samples. The percentage of the isolates as follows: EAEC (25.9%), EPEC (16.3%), ETEC (12.9%), STEC (1.7%), EIEC (0.6%), DEC co-infections 42.6%. *In Sao Paulo, Brazil, Lieobchen et al* (2011) studied the prevalences of different pathotypes among 702 *E. coli* isolates from Brazilian patients with diarrhea using multiplex PCR. Most were EAEC strains (78.0%) followed by ETEC (11.9%) and EHEC (3.4%). *In a prospective study conducted by Nures et al* (2011) on 250 children with diarrhea in Teresina, Brazil, ETEC strains were isolated from (9.2%) of children. The isolated strains were found susceptible to
ciprofloxacin, nalidixic acid and resistant to ampicillin, cephalothin and co-trimoxazole. \(^{(67)}\)

Under the title: Enteroinvasive *E.coli* infection in Sudan, Omer and Ibrahim in the year (1983), examined 223 stool specimens and rectal swabs for EIEC using Sereny test. EIEC was found in 10 (4.5\%) specimens. \(^{(68)}\)

**1-1-3-2. Shigella**

The genus *Shigella* is a member of the family Enterobacteriaceae and is closely related to the genus *E.coli*. *Shigella* species are not members of normal gastrointestinal flora of humans or animals. According to their antigenic structure, *Shigellae* are classified into four groups which include:

Group A *Shigella dysenteriae*, group B *Shigella flexneri*, group C *Shigella boydii* and group D *Shigella sonnei*. They are the causative agents of bacillary dysentery or shigellosis. \(^{(69,70,71,72)}\)

Shigellosis still continues to be an important global problem. The annual number of *Shigella* episodes throughout the world was estimated to be 164.7 millions of which 163.2 million were in developing countries (with 1.1 million deaths and 1.5 million in industrial countries). \(^{(73,74,75,76)}\)

**1-1-3-2-1. Epidemiology of diarrhea caused by Shigella:**

In the study conducted by Lwalokun et al. (2001), 62 *Shigella* species were isolated from 459 patients with diarrhea, in Lagos, Nigeria. *Shigella flexneri* accounted for 51\% of the total number of *Shigella* isolated. All isolated strains were sensitive to ciprofloxacin and ofloxacin. Over 70\% of the isolates were resistant to ampicillin and co-trimoxazole. Twenty one different multi-drug resistance pattern were observed in these isolates.\(^{(77)}\)

In Sudan (2002) Musa Abdalla et al studied *Shigella* gastroenteritis in Khartoum State. He reported that 16 *Shigella* species were isolated from 100 (16\%) stool specimens; in which *S.flexneri* showed predominate presence (62.5\%) followed by *S.boydii* (25\%), *S.dysenteriae* (6.25\%) and *S.sonnei* also (6.25\%).
The antimicrobial susceptibility pattern of the isolated *Shigella* in this study showed that all isolates were susceptible to ciprofloxacin and 60% were susceptible to co-trimoxazole.\(^{(78)}\)

In Kenya, Brooks et al. (2003) conducted a study to characterize the epidemiology of bloody diarrhea. From 451 stool specimens, 231 (51%) yielded 247 bacterial pathogens. *Shigella* species were the most common isolates 198 species, 97 of which were *S.*flexneri followed by 80 *S.*dysenteriae, 13 *S.*boydii and 8 *S.*sonnei. Campylobacter was isolated from 33 specimens and non-typhoidal Salmonella from 15 specimens. More than 90% of Shigella and Salmonella isolates were resistant to co-trimoxazole, tetracycline and more than 80% were resistant to ampicillin.\(^{(79)}\)

Moez et al. (2003) carried out a study to determine the prevalence and pattern of antimicrobial resistance of *Shigella* species among patients with acute diarrhea in Tehran. From 734 stool samples collected, 123 yielded *Shigella* spp. (16.8%). Of the *Shigella* isolates 90.8% were resistant to one or more antimicrobial agents and 87% were multi-drug resistant.\(^{(80)}\)

In Sudan, an outbreak of shigellosis occurred in the year 2004 in the state of North Darfur in the Abu Shoak camp, covering a population of approximately 40,000 people. About 1340 cases of bloody diarrhea were detected with 11 deaths reported.\(^{(81)}\)

Zafar et al. (2005) carried out a study to assess the frequency of serogroups and serotypes, as well as the antimicrobial susceptibility pattern of *Shigella* species isolated from stool samples received from children with diarrhea and dysentery in Karachi, Pakistan. Out of 4688 stool samples, 193 (4.1%) were positive for *Shigella* species. *S.*flexneri was the predominant serogroup (58%) and *S.*dysenteriae was the least common (11%). All isolates were susceptible to ceftriaxone and ofloxacin. A high rate of resistance was observed with co-trimoxazole (87.75%) and ampicillin (55.5%).\(^{(82)}\)

Tema et al. (2007) determined the frequency and antimicrobial susceptibility pattern of *Shigella* spp. isolated from 489 patients with bloody diarrhea in Mwaza City, Tanzania. *Shigella* species were isolated
from 69 (14%) stool specimens, 62 (90%) were *Shigella flexneri* and 7 (10%) were *S. dysenteriae*. All isolates (100%) were susceptible to ciprofloxacin and gentamicin. Most isolates showed high resistance to tetracycline, ampicillin and co-trimoxazole.\(^{(83)}\)

In the study done by Jafari in Tehran, Iran (2008). 808 patients with acute diarrhea were enrolled. A total of 369 (45%) bacterial pathogens were recovered. Results showed *Shigella* species were 155 (45.6%), diarrhoeagenic *E.coli* were 143 (38.8%), *Salmonella* spp. were 51 (13.8%) and *Campylobacter* spp. were 20 (5.4%).\(^{(84)}\)

Walid Ahmed (2008) conducted study on bloody diarrhea among children in Khartoum State. He reported that 114 (26.4%) bacterial pathogens were isolated from 432 stool specimens. The most frequent isolates were *S. flexneri* 33 (7.6%) followed by EIEC (5.9%), *S. dysenteriae* (3%), *Shigella sonnei* (2.8%), non-typhoid *Salmonella* (2.8%), *S. boydii* (2.1%), *Campylobacter* spp. (1.2%), EPEC (0.9%) and EHEC (0.6%).

The antimicrobial activity of the isolates against some routinely used antimicrobial agents showed that all isolates were susceptible to ciprofloxacin followed by gentamicin (96.4%) while (63%) were susceptible to co-trimoxazole.\(^{(85)}\)

**1-1-3-3. Salmonella:**

Salmonellae are gram negative rods, and members of the family Enterobacteriaceae. Currently, there are more than 2500 serotypes of *Salmonella* based on O and H antigens. Few of these serotypes are from human sources and they are the causative agents of enteric (typhoid) fever.\(^{(4,86,87)}\)

The majority of these strains are found in animals including poultry, livestock, reptiles and pets. These strains are causative agents of gastroenteritis and known as non-typhoidal Salmonellae. Non-typhoidal Salmonellae are major bacterial pathogens of food-borne infection in both developed and developing countries. In most parts of the world, *Salmonella typhimurium* and *Salmonella enteritidis* are the commonest strains.\(^{(88,89,90,91,92)}\)
The illness is characterized by mild fever, vomiting, abdominal pain and diarrhea. Also, non-typhoidal Salmonellae are causative agents of some extra intestinal diseases, particularly in HIV infected individuals and African children. \(^{(93,94,95,96)}\)

1-1-3-3-1. Epidemiology of diarrhea caused by Salmonella:

The aetiology of diarrhea in children in a low-income housing project in Bangkok, Thailand was determined by Varvithya.w. et al in (1990). The following isolates were reported: non-typhoidal Salmonellae (13%), *Campylobacter jejuni* (12%), Rotavirus (12%) Enterotoxigenic *E.coli*, (7%) Shigella (6%) and Enteroinvasive *E.coli* (1%). \(^{(97)}\)

Ling and Cheng et al (1993) studied the role of enteric pathogens in patients with diarrhea in Hong Kong. Out of 3267 organisms isolated, the gastroenteric Salmonella were the most common pathogens (45%) followed by rotavirus (34%) and Campylobacter (11%). \(^{(98)}\)

A similar study was conducted by Samonis et al (1997) in the Island of Crete. From 826 patients, Salmonella species were the most frequently isolated organisms (in 13.6% of patients) followed by Campylobacter (in 4.7%) and EPEC (in 3.9%). \(^{(99)}\)

In Bahrain, Ismaell et al (2002) investigated enteropathogens in children with diarrhea. Eight-hundred and five faecal samples were cultured for Salmonella, Shigella, Campylobacter and EPEC. Eighty-three (10.3%) samples were found positive for Salmonella (46 isolates), Shigella (26 isolates) *Campylobacter jejuni* (7 isolates), and EPEC (4 isolates). \(^{(100)}\)

In India Taneja et al (2004), investigated 1802 faecal samples for common bacterial enteropathogens and their antibiotic susceptibility pattern. Stool samples were cultured and identified by standard methods. Antibiotic susceptibility was done by Stocke’s disk diffusion method. Stool specimens from 119 patients yielded Shigella, Salmonella, *Vibrio cholerae* and Aeromonas. Shigella sp. was commonest followed by non-typhoidal Salmonella (27), *Vibrio cholerae* 01 (19), Aeromonas species (14) *Salmonella typhi* and *paratyphi A* (2 isolates each). Resistance to antimicrobial agents was common among all pathogens. Seven isolates of Shigella were resistant to ciprofloxacin while 63.6% were resistant to co-trimoxazole, 18.5% of non-typhoidal Salmonella were resistant to
ciprofloxacin. *Vibrio cholerae* were generally susceptible to tetracycline (only 1 isolate of 13 was resistant) but showed high resistance to co-trimoxazole (77.8%).\(^{(101)}\)

Rollo et al in Italy (2004) studied 198 hospitalized persons for acute gastroenteritis. Non-typhoidal Salmonella was isolated from stool of 49 them. Their antimicrobial susceptibility tests were performed by agar disk diffusion method, among all antimicrobials tested, only fluoroquinolones showed susceptibility in vitro in all strains.\(^{(102)}\)

Kariuki et al in Kenya (2006) studied non-typhoidal Salmonella (NTS) isolates from pediatric admission at two hospitals in Nairobi, and followed the index cases to their homes, where rectal swabs and stools from parents and siblings, and from animals in close contact, were obtained. The majority of NTS obtained from cases were *Salmonella enterica* serotype *Typhimurium* 106 (54.9%) out of 193 and *Salmonella enterica* serotype Enteritidis 64 (33.2%). A significant proportion (34.2%) of which were multiple resistant to three or more antibiotics including ampicillin, tetracycline co-trimoxazole and chloramphenicol. Only 23.4% of NTS were fully susceptible to all 10 antibiotics tested. Of the 32 NTS obtained from contacts (nine adults and 23 children) at the homes of index cases, 21 (65.6%) isolates were similar by antibiotic-susceptibility profiles and plasmid content. Only 3 out of 180 (1.7%) samples from environmental sources, including animals, soil, sewers and food contained NTS matching those from corresponding to index cases.\(^{(103)}\)

In Canada Mataseje et al (2009), determined the antimicrobial susceptibility tests for 6692 non-typhoidal Salmonella isolates. Cefoxitin resistance was observed in 283 of the isolates. The common isolates were *S. typhimurium* and *S. newport*.\(^{(104)}\)

In Sudan, isolation of Salmonella was reported by different investigators. Mamoun et al (1992) isolated 21 Salmonella strains from several poultry farms in three different states in Sudan. All strains were found to be *S. enteriditis*.\(^{(105)}\)
Elsafi et al (2009) reported the isolation of 4 Salmonella isolates from 119 feacal samples (3.4%) collected in Khartoum state. Two isolates were serotyped as *S. droganna* and the other two as *S. umbadah*.\(^{106}\)

1-1-3-4. **Campylobacter**:  
Campylobacters are microaerophilic, curved, S-shaped or spiral shape Gram negative rods primarily zoonotic with a variety of animals implicated as reservoirs for infection which include poultry, cattle, sheep and pigs.\(^{107,108,109}\) Campylobacter species are leading causes of diarrhoeal illness worldwide with as many as 400 million cases annually.\(^{110,111,112}\)

There are at least 18 Campylobacter species, of which *C. jejuni* and *C. coli* are most important agents of Campylobacter enteric infections. Transmission of the organism has been associated with the consumption of unpasteurized milk, undercooked meat and contaminated water. Symptoms and signs usually include fever, abdominal cramping and diarrhea with or without blood or white blood cells. Dysentery like diarrhea is most common. Infection is usually self-limiting, but relapse may occur in 5 to 10% of untreated patients. Also Campylobacter infection may mimic acute appendicitis and may result in unnecessary surgery.\(^{113,114,115,116}\)

1-1-3-4-1. **Epidemiology of diarrhea caused by Campylobacter**:  
Ashraful and Kazi in Bangladesh (1991) investigated the importance of *C. jejuni* as an aetiological agent of childhood diarrhea in an urban children hospital in Dhaka over a period of 1 year. *C. jejuni* was isolated from 102 (25.5%) patients with diarrhea. No *C. coli* was detected.\(^{117}\)

Zaman in Saudi Arabia (1992) carried out a study on the incidence of Campylobacter infection in 1217 patients with diarrhea. Campylobacter was isolated from 55 (4.5%) patients second in prevalence to Salmonella (6.2%). Shigella were isolated from 4.2% of patients. Analysis of the results showed that 69% were *C. jejuni* and (31%) *C. coli*.\(^{118}\)

Fahey T. et al in Oxford, England (1995) reported an outbreak of *C. jejuni* enteritis associated with failed milk pasteurization. The outbreak of this infection was found to have been associated with consumption of inadequately pasteurized milk from a local dairy.\(^{119}\)
A prospective study over a period of 12 months was carried out by Gedla and Asheffa (1996) to know the incidence of Campylobacter enteritis among children visiting the outpatient department of a teaching hospital in north-west Ethiopia. Campylobacter species were isolated from the stool of 60 children out of 434 (13.8%). The highest isolation rate was found in children from 6 to 24 months.\(^{(120)}\)

Ugsal et al (1997) in Turkey, studied 400 children with diarrhea to assess Campylobacter isolation rate in childhood acute gastroenteritis. *Campylobacter jejuni* was found to be the second most common isolate with a rate of 8.3%. The first being Shigella strains.\(^{(121)}\)

The first isolation of Campylobacter species in Sudan was reported by Mansour in the year (1994) in her study of bacterial causes of diarrhea in Sudanese children. The study revealed that the most common isolated pathogens were Campylobacter species.\(^{(122)}\)

Eltoum in the year (1998) investigated 50 stool specimens from Sudanese patients. He isolated 2 Campylobacter species from 2 patients which represented (4%) of the total specimens.\(^{(123)}\)

At the end of the same year (1998), bloody diarrhea in Sudanese children was examined by Abdalla. She reported that Campylobacter species were among the main causative agents of bloody diarrhea in Sudanese children.\(^{(124)}\)

Also in Sudan in the year (2000) Elamin Ibrahim isolated 4.5% of Campylobacter species from 200 stool specimens in his study about Campylobacter gastroenteritis in Sudan.\(^{(125)}\)

### 1-1-3-5. *Aeromonas*:

Members of the genus *Aeromonas* are medically important oxidase-positive gram negative rods and are ubiquitous in aquatic environment worldwide. It is also found in a wide variety of fresh produced meat and dairy products. At least 15 species of *Aeromonas* are known, of which *A. hydrophila*, *A. sobria* and *A. caviae* are most important species associated with clinical specimens.\(^{(25,109,126)}\)

*Aeromonas* species have been documented in a variety of human infections, including septiceamia, meningitis, wound and lung infections, although the most frequent reports indicate the increasing association of
Aeromomas species with acute gastroenteritis and traveller’s diarrhea. Aeromomas gastroenteritis ranges from an acute watery diarrhea (most common form) to dysenteric illness to chronic illness. Infections are usually self-limiting, but children may require hospitalization due to dehydration. (127,128,129,130)

Recent studies indicated that Aeromomas-associated diarrhea is sporadic, similar to infection caused by Vibrio cholerae non-01 and non-0139, serogroups. The pathogenesis of Aeromonas infection is complex and multifactorial. Aeromonas species can produce a variety of virulence factors, including cytotoxins, enterotoxin and haemolysins. (131,132,133) Aeromonas species can grow on most types of selective media used for isolation of enteric organisms and are most easily confused in the laboratory with other oxidase-positive organisms, particularly Vibrios and Plesiomonas. Ampicillin blood agar is a good selective medium used for isolation of Aeromonas from stool specimens. (24,25,134)

1-1-3-5-1. Epidemiology of diarrhea caused by Aeromonas:

Kumarasinghe et al (1992) in Singapore studied the prevalence of bacterial agents of diarrheal diseases in 4508 stool specimens. Nontyphoidal Salmonella were isolated from (10.8%) specimens followed by Campylobacter (1.9%) and Aeromonas hydrophila (1.8%). (135) In Bangladesh Hossain et al (1992) studied the incidence of Aeromonas in children with diarrhea. They investigated 305 stool samples from children with diarrhea and equal number of specimen from non-diarrhoeal control children. Aeromonas species were isolated from 37 (12.1%) diarrheal and 5 (1.6%) control cases. Out of 37 diarrheal isolates 13 (35%) were Aeromonas hydrophila, 19 (51%) Aeromonas sorbia and 5 (13.5%) Aeromonas caviae. (136)

In a retrospective study (1993) Reina-et al in Brazil investigated 7653 stool specimens from patients with gastroenteritis. Aeromonas species were isolated from 282 (3.7%) of the specimens. (137) Obi-cl. et al in Nigeria (1997) investigated 1200 stool specimens for the prevalence of bacterial causes of diarrhea. The study showed high prevalence of diarrhoeagenic E.coli strains and Campylobacter. Aeromonas isolates constituted 5%. (138)
Sathiekal et al in Thailand (2001) investigated 152 stool specimens for the incidence of bacterial diarrhea in AIDS patients. They reported the following results about the bacterial isolates: Salmonella group B 12 (19.7%), *Plesiomonas shigelloides* 44(27%), Aeromonas species 29 (19%) \(^{(139)}\).

Malttezou et al (2001) carried out a prospective study to describe the epidemiology of acute diarrhea in children from an outpatient setting in Athens, Greece. From 132 stool specimens, enteropathogens were detected in 66 (48%), isolates included Rotavirus from 19 patients, Salmonella species from 12, Campylobacter species from 10, Aeromonas species from 9, EPEC from 6, *Yersinia enterocolitica* from 2 and Shigella spp. from 1 patient. Adenovirus and *Giardia Lamblia* were detected in 10 patients. \(^{(140)}\).

In a study carried out in Cuba to analyze the possible pathogenic role of Aeromonas spp. in children with acute diarrhea, Bravo et al in (2012) reported the isolation of 166 (7.15%) species of Aeromonas from 2322 stool specimens. The Aeromonas spp. more frequently isolated were *A. caviae, A. hydrophila* and *A. sobria*. \(^{(128)}\)

1-1-3-6. *Yersinia*:
These are Gram negative cocco-bacilli, member of the family Enterobactericeae. There are more than 10 species of *Yersinia* of which *Yersinia pestis* and *Yersinia enterocolitica* are usually associated with human infections. *Yersinia enterocolitica* can be found as animal parasites and as saprophytes in water soil and vegetation. It is becoming increasingly identified as a cause of food-borne gastroenteritis. \(^{(141,142,143)}\)

Human disease may result from ingestion of contaminated food or from contact with environment. Man-to-man transmission also can occur. The infection is characterized by acute abdominal pain, profuse (sometimes bloody) diarrhea and headache. \(^{(144,145,146)}\)

*Yersinia enterocolitica* can grow on most selective media used for isolation of enteric pathogens including MacConkey, DCA and SS agars as small colourless non-lactose fermenting colonies, but the best selective
medium for isolation of *Y. enterocolitica* from stools is known as Cefsulodin-Irgasan- Novobiocin agar (CIN). (25,147,148)

1-1-3-6-1. Epidemiology of diarrhea caused by *Yersinia enterocolitica*:
In Atlanta, United States (1991) after an outbreak of *Yersinia enterocolitica* infection among black children, Lee et al. and his colleagues investigated 4841 stool specimens for different enteric pathogens to determine the importance of this pathogen in other communities with large black population. *Yersinia enterocolitica*, Shigella, Campylobacter and Salmonella were identified in 38, 48, 60 and 98 specimen respectively. (149)

Nauvi et al from St.louis University in the year 1993 studied *Yersinia enterocolitica* enteritis in children. They reported the isolation of 4 (2.8) *Yersinia enterocolitica* from 70 stool specimens. (150)

In Porto Vello, Brazil (1996) Orlandi et al conducted study on the enteropathogens associated with diarrheal diseases. They found that rotavirus were the most common (19.2%) followed by Shigella (6.15%), Salmonella (6.9%) EPEC (3.1%) and *Yersinia enterocolitica* (0.8%). (151)

Vaida et al in Brazil (1996) studied 8363 subjects with acute diarrhea for the role of Salmonella, Shigella and Yersinia in acute diarrheal disease. They isolated enteric pathogenic organisms from 486 (5.81%) cases; as follows Shigella (69.13%), Salmonella (27.78%) and *Yersinia enterocolitica* (3.08%). The serogroups of Shigella most often isolated were *Shigella sonnei* in 196 patients (35.11%). Regarding the cases with Salmonella, in most of the cases *Salmonella enteritidis* was isolated (60.74%) followed by *Salmonella typhimurium* and other Salmonellae from serogroup BO (23.70%). (152)

Andualem and Geyid (2003) carried out a study to determine and describe the prevalence of diarrhea caused by *Yersinia enterocolitica* isolates in comparison with the commonly encountered diarrhaegenic Salmonella and Shigella among all age groups in out-patients of Addis Ababa, Ethiopia. Standardized bacteriological isolation and biochemical test techniques were used. Among the stool samples of 205 patients tested for bacteriological culture, only 3 (1.5%) were positive for
Yersinia enterocolitica, 22 (10.7%) for Salmonella and 12 (5.8%) for Shigella. (153)

1-1-3-7. Morganella morgani:-
These are Gram-negative rods, members of the family Enterobacteriaceae and are frequently found in human and animal intestines and in sewage, soil and water. It is associated with nosocomial infections including urinary tract, pneumonia, wound infections and bacteraemia. (69,154,155) It has been isolated from diarrheal stools in the absence of other known bacterial enteropathogens. Also it has been reported as causing diarrhea in infants. (25,147,156,157)

1-1-3-7-1. Epidemiology of diarrhea caused by Morganella morgani:
The pathogenic role of Morganella-Proteus-Providencia group of bacteria in human faeces was examined by Muller in (1986). He investigated a total of 2693 specimens, with 1422 from healthy persons and 1271 from patients suffering from enteric diseases to isolate species of the Morganella-Proteus-Providencia group and to evaluate the role of these bacteria in intestinal disorders. Morganella morgani occurred more frequently in diarrhoeic cases (17) than in healthy people 4 only. The difference is statically significant (p<0.01). The results of this study indicated that Morganella morgani have a role in diarrheal diseases. (158)

A prospective study was carried out by Ahren et al (1990) in University of Goteborg, Swedan in 94 Swedish travellers to Singapore, Hong Kong and Japan. Ninty-four stool specimens were analysed for Salmonella, Shigella, Yersinia, Campylobacter and other enteropathogens. A potential enteropathogens were identified in (30%) of the participants. The most common isolates were ETEC (14%) followed by Salmonella (11%) and Campylobacter (7%). Six strains of Morganella morgani were identified from the primary cultures of all specimens. (159)

Hsuan et al (2012) in the Tri-service Hospital-Taiwan reported a case of 22-year old man who presented with a 3-day history of watery diarrhea, abdominal pain and fever. The result of blood culture performed on admission showed the presence of Morganella morgani. The isolated organism found to be susceptible to amikacin, gentamicin, ciprofloxacin, ceftriaxone, ceftazidime, cefipime and imipenem. (160)
1-1-3-8. Vibrios:
Vibrios are oxidase-positive, Gram-negative, curved rods found in marine environment, fresh, brakish water, shellfish and other seafoods. The genus comprises 35 well-defined species. *Vibrio cholerae* and *Vibrio parahaemolyticus* are the most common species associated with human infections, although man is the main reservoir of *Vibrio cholerae*. *Vibrio cholerae* possesses at least 193 different O-antigens, on which basis it has been divided into corresponding numbers of serogroups. Epidemic cholera is caused only by serogroups 01 and 0139. (67,153,161)

Cholera is an endemic disease in Asia where the rate of infection doubles during the rainy season. It is estimated that tens of thousands of people in the world are affected every year due to cholera outbreaks and epidemics. (162,163)

1-1-3-8-1. Non-cholera vibrios:
Other serogroups of *Vibrio cholerae* are known as Non-cholera Vibrios (NCV), also known as *Vibrio cholerae* non-01/non-0139. They resemble *Vibrio cholerae*-01 biochemically and genetically. Most strains are associated with non-epidemic diarrhea and some extraintestinal diseases. They cause mild, sometimes bloody diarrhea, often accompanied by abdominal cramps. *Vibrio cholerae* non-01 may elaborate a wide range of virulence factors including enterotoxins, cytotoxins and haemolysins. Few strains can produce cholera toxin. They can be isolated and characterized by similar techniques used for *Vibrio cholerae* 01. (164,165,166,167)

1-1-3-8-2. Epidemiology of diarrhea caused by non-cholera Vibrios:
Notario et al (1996) studied 570 stools specimens for enteropathogenic agents associated with acute diarrheal disease in Rosario, Argentina. They reported that EPEC was the most prevalent agent affecting 144/570 (25%) followed by Shigella species (8.8%). Campylobacter species were found in (6.1%) of patients, Salmonella in (2.8%). There was (1.8%) of Aeromonas spp., and only two cases due to *Vibrio cholerae* non 0-1. (168)

In a study carried out by Torres et al (2001) for organisms associated with infantile diarrhea in Montevideo, 256 stool specimens were
investigated using updated procedures. Potential pathogens were found in (63.8%). EPEC were the most common isolates followed by Campylobacter and Shigella spp. Only one Vibrio cholerae non-01 was isolated. (169)

1-1-4. Laboratory diagnosis of bacterial diarrhea:-

The laboratory diagnosis of bacterial diarrhea depends mainly on isolation and identification of these agents from stool specimens. Also many serological techniques are used for detection of these agents from stool and sometimes from blood. Recently modern molecular biological techniques are used for identification and characterization of the genes of these organisms. (13,14,147,195)

1-1-5. Antimicrobial treatment:-

Most diarrheal diseases are self-limiting and does not need antimicrobial treatment. The wrong choice of antimicrobial agents will worsen the symptoms of gastrointestinal infections which may change intestinal microflora, promote the emergence of resistant strains and overgrowth of potential pathogenic bacteria and fungi. Risks and benefits should be considered before prescribing antimicrobial agents, but it has been documented that antimicrobial agents improve the diarrhea associated with cholera, shigellosis, enteric fever and traveller’s diarrhea caused by enterotoxigenic E.coli. (170,171,172)

The WHO programme for control of Diarrheal Diseases “Geneva” recommended that all infants and children with bloody diarrhea should be treated promptly with an antimicrobial effective against Shigella such antimicrobials include co-trimoxazole, nalidixic acid, newer quinolones and ceftriaxone. (173)

Quinolones are recommended for the treatment of acute diarrheal disease because they are active in vitro against most organisms that cause bacterial diarrheal diseases and they achieve high stool concentration. (174)

1-1-5-1. Antimicrobial resistance:

Studies from many countries have reported resistance of Campylobacter, Shigella, E.coli and Salmonella to several classes of antimicrobial agents.
In many geographic areas of the world, resistance patterns have demonstrated a consistent increase over the course of time. The antimicrobial resistance among species of Salmonella, Shigella, Esherichia and Aeromonas isolated from children with diarrhea in 7 Argentinian Centers, was studied by Binsztein et al in the year (1999). They reported that diarrheagenic E.coli exhibited (74.5%) of resistance against ampicillin, (64.2%) against co-trimoxazole and Shigella species, (62%) and (75.6%) respectively. Salmonella species showed (35%), (14%), (41.8%), (65.4%), (14.5%) and (13.6%) resistance against ampicillin, chloromphenicol, co-trimoxazole, sulfadiazin, gentamicin and fosformycin respectively. Aeromonas showed significantly lower resistance percentage. A retrospective 6-month period study, carried out by Kaminski and Bogomolski (1994) from Israel, analyzed the results of stool culture from 209 patients with acute diarrhea admitted to the emergency room of Hadassah University Hospital. Seventy-eight cultures (37%) were positive for one or more bacteria. Shigella was the most commonly isolated pathogen (68%); Shigella sonnei compromised (72%) and Shigella flexneri (19%) of all the bacterial isolates. While no antimicrobial resistance to ciprofloxacin was found for both Shigella species, only (36%) and (26%) of Shigella isolates were sensitive to ampicillin and co-trimoxazole respectively. Fagarasan et al (1997) in the University of Medicine and Pharmacy, Cluj-Napoca, investigated the resistance patterns, plasmid profiles and genetic resistance. They determined that 38 isolates of Salmonella enteric serotype Typhimurium and 19 isolates of Salmonella enterica serovar Enteriditis derived from stool of hospitalized children of plasmid and antibiotic resistance was very high in Salmonella typhimurium isolates. They were susceptible only for third generation cephalosporins and fluoroquinolones. Salmonella enteritidis isolates were susceptible to many antibiotics, except tetracycline and co-trimoxazole. In a study carried out in India by Ballac et al (1998), 531 stool samples were investigated for Shigella species. Twenty-nine (5.46%) different Shigella species were isolated, out of them S. flexneri were (16.17%) S.
boydii 8 (27.58%), S. dysenteriae 3 (10.34%) and S. sonnei 2 (6.89%). The antimicrobial susceptibility pattern was determined for all isolates against three fluorinated quinolones, nalidixic acid, ciprofloxacin and norfloxacin. Five strains of S. flexneri and one strain of S. dysenteriae were found to show resistance to the three antimicrobials used. (179)

In Sudan, Ahmed et al (2000) performed antimicrobial susceptibility tests on 497 bacterial isolates from Sudanese patients with diarrhea or urinary tract infections. Shigella dysenteriae type 1 and EPEC showed high resistance rates against the commonly used antimicrobial agents: ampicillin, amoxycillin, chlormphenicol, tetracycline, co-trimoxazole and neomycin. These were completely susceptible to ciprofloxacin. (180)

In study carried out by Panhotra et al (2004) in Saudi Arabia, 524 strains of NTS were isolated from faecal samples. Resistance to ampicillin was observed in 22.9% and cotrimoxaxole in (18.5%) of strains. Nalidixic acid resistance was encountered in (9.9%) and ciprofloxacin in (2.3%). (181)

Chiu and Lin (2004) in Taiwan, determined the antimicrobial susceptibility pattern of 66 Salmonella species isolated from blood and stools of children in Chang Gung children’s Hospital. They reported that all isolates were susceptible to third generation cephalosporins, while high resistance rates were reported for ampicillin (85%) and co-trimoxazole (81%). Ciprofloxacin resistant strains constituted (49%). (182)

In Sousse, Tunisia (2005), Bouallegue et al., studied an outbreak of Salmonella enterica serotype Livingstone occurred in neonatal ward-Farhat Hached Hospital. They reported that 16 isolates of Salmonella from hospitalized babies with diarrhea demonstrated resistance to ceftriaxone and ceftazidime due to the production of extended spectrum beta-lactamases (ESBLs). The isolates were also resistant to aminoglycosides kanamycin, gentamicin and amikacin and co-trimoxazole. (183)

Jain et al in India (2005) carried out a prospective case-control study from July to December 2002 to investigate the prevalence and socio-epidemiological determinants of Campylobacter infection in a rural community in north India and the resistance of isolated strains. From 348
stool specimens, Campylobacter species were isolated from 47 (13.5%) subjects. Antibiotic resistance of Campylobacter species showed: ampicillin (81.6%), ciprofloxacin (71.4%), tetracycline (26.5%) furazolidine (14.3%), gentamicin (10.2%) and erythromycin (6.1%), (30.6%) of strains were multi-drug resistant. (184)

1-1-6. Molecular techniques:
There is a considerable improvement in the detection rate of enteric organisms from stool specimens using rapid, highly sensitive methods such as PCR and other modern techniques in comparison with conventional culture and serological techniques. (185,186)
Lejima et al (2004) in Japan conducted a study to compare between PCR and conventional culture methods in the detection of diarrheagenic bacteria in human stool specimens. They reported that from 40 stool specimens 16 (40%) were found to be positive by conventional culture methods while 28 (70%) were found to be positive using real-time PCR assay. (186)
A similar study was conducted by Amar et al (2004) in London. They investigated 92 faecal samples collected from patients with community-acquired diarrhea by conventional methods and PCR-based techniques. Aetiological agents were detected in (15%) of samples using conventional methods, while those detected by PCR reach up to (41%) of samples. (187)
Kozub E. et al in Germany (2007) investigated 861 stool samples from children with diarrhea for the prevalence of traditional and emerging types of EPEC and EAEC strains and to characterize their virulence genes. Serological and PCR-based methods were used for detection and isolation of these strains. Agglutination with traditional EPEC and EAEC O-group specific anti-sera resulted in detection of 38 strains; 26 of these carried virulence factors of EPEC and EAEC. PCR screening for eae gene resulted in isolation of 97 strains. The 97 EPEC and EHEC strains were divided into 36 O-serogroups and 21 H-types. Only nine 9 strains belonged to the traditional EPEC O-groups 026-055-086 and 0128. In contrast, EPEC serotypes 028: H28-051:H49-0115:H38 and 0127: H40 were found in multiple cases.
1-1-7. Vaccination:-
Diarrheal disease is a major public health problem worldwide, leading to malnutrition, susceptibility to other infections and death, especially in developing countries. Prevention of diarrheal disease by improved hygiene and provision of sanitation and water treatment is impractical in most developing countries, where morbidity and mortality rates are highest. For this reason, development of vaccines against most important gastrointestinal infections remains a high priority.\(^{(188,189,190)}\)

Levine and Noriega (1993) in the center of vaccine development, University of Maryland, reported that there has been considerable progress made in developing vaccines against the enteric infections of greatest public health importance, including vaccines against Rotaviruses, Salmonella causing typhoid fever and *Vibrio cholerae* 0-1. Several candidate vaccines against *Shigella* and enterotoxigenic *E.coli* are on clinical trials.\(^{(191)}\)

Lassuro et al (2005) carried out a study to evaluate a prime-boost vaccine regimen against ETEC. They reported that this vaccine conferred protective immunity against ETEC.\(^{(192)}\)

A similar study was conducted by Steffen et al (2005) to evaluate an oral whole-cell vaccine against LT toxin of ETEC. They reported that this vaccine demonstrated protection for more than 3 months after vaccination.\(^{(189)}\)
1-1-8. Rationale :-

Acute bacterial gastroenteritis is among the most frequent diseases worldwide. Diarrhoea, which is the most common outcome of these diseases, remains a leading cause of childhood morbidity and mortality in many parts of the world, especially in Africa, Asia and South America.

The list of bacteria known or suspected to cause diarrheal diseases in humans is a long one, and organisms are constantly being added to it. In the last two decades, additional aetiological agents have been identified worldwide. Most of these agents are not reported in the data of most hospitals records in Sudan.

A definitive identification of organisms that cause diarrhea requires many procedures that include culture, serology and modern molecular biological techniques. Many of these procedures are expensive and need facilities which are not available in most of the routine laboratories in Sudan.

The lack of facilities or shortage of some information in many routine and teaching laboratories in Sudan lead physicians and microbiologists excluding important etiological agents of diarrhea, although some of these agents have bad consequences and may lead to life threatening diseases e.g. haemolytic uraemic syndrome that is caused by Enterohaemorrhagic E.coli. 0157

The antimicrobial susceptibility profile of many of these agents is not known, although there is a high incidence of the development of resistant strains worldwide.
Objectives:-

1-1-9. General objectives:
To determine the bacterial causes of diarrhea in Sudanese patients and their antimicrobial susceptibility in Khartoum State

1-1-9-1. Specific objectives:-
1- To isolate aerobic bacteria that causes diarrhea from stool specimens using a wide range of selective media.
2- To identify the isolated pathogens using conventional biochemical methods & API system techniques.
3- To confirm these identification techniques by serological tests.
4- To detect the presence of any aetiological agents that have not been reported in Sudan before.
5- To assess the antimicrobial susceptibility pattern of the isolated strains against some of the routinely used antimicrobial agents for the treatment of gastroenteritis in Sudan.
CHAPTER TWO

Materials and methods

2-1. Study Design:-

Prospective, cross-sectional, hospital based study.

2-2. Study area:-

Omdurman Military Hospital

Khartoum Peadiatric Emergency Hospital

Modern Medical Centre - Khartoum

Prof. El-Kadru and Elamin Ibrahim- Private Clinic, Khartoum North

The related hospitals are referral and educational hospitals, including different specialties, serving various patient groups and covering most population of different areas.

2-3. Study population:-

Both adults and children presenting with diarrhea who attended the above mentioned study area.

2-4. Sample size:-

A total number of 900 stool specimens collected from patients were included in this study.

2-5. Study duration:-
This study was conducted over 3 years from Jan. 2009 to Feb. 2013.

2-6. Data analysis:-

Statistical package for social sciences (SPSS) version 16, along with Microsoft Excel committee.

2-7. Ethical consideration:-

Permission to conduct this study was obtained from hospital authorities and informed consent was obtained from the parents/guardians of every patient before taking the stool samples. The study protocol was approved by the Ethical Committee of University of Gezira

2-8. Specimen collection and transportation:-

Stool specimens were collected in wide mouth disposable plastic containers, then fecal swabs were taken from the specimen and submitted in Cary-Blair transport medium. (Appendix 1-A)

2-9. Working area:

All specimens were delivered to the microbiology laboratory of the Faculty of Medical Laboratory Sciences, University of Khartoum

2-10. Macroscopical examination:

Stool specimens in the plastic containers were examined macroscopically and reported for its consistency, colour and the presence of any abnormal components, such as mucus and blood.
2-11. Microscopical examination:

A wet-mount of each specimen was prepared in 0.05 % methylene blue in normal saline on a clean slide and examined under the microscope for the presence of white and red blood cells.

2-12. Culture:-

The swab containing stool specimen was taken from the transport medium using sterile forceps then used for inoculating different types of selective media which included:

DCA (Appendix 1-B), XLD (Appendix 1-C) ,HE (Hektoin Enteric Agar) (Appendix 1-D) ,MacConkey agar No.3 (Appendix 1-E) ,TCBS (Appendix 1-F) and Campylobacter selective media (Skirrow formulation) (Appendix 1-G)

2-13. Incubation:-

All inoculated plates, except Skirrow plates were incubated aerobically at 37°C overnight.

Skirrow plates were incubated at 42°C up to 48 hours under microaerophilic conditions which was achieved by anaerobic jar with microaerophilic gas-generating kits Oxoid CampyGen (Appendix 2-A) and also by candle jar.(193)

2-14. Identification:-

At the end of the incubation period, plates were examined for growth of suspected colonies.
The following colonies were picked for identification procedures:

Non-lactose fermenting colonies from DCA (colorless), XLD (pink) and HE medium (blue green)

Lactose fermenting colonies from MacConkey agar No.3 (pink)

All yellow or green colonies from TCBS

All colonies from Skirrow plates

All selected colonies were subcultured on MacConkey agar plates for purity. (193,194,195)

2-14-1. Gram stain:-

Smears were prepared from the purity plates, stained by Gram method and examined microscopically using light microscope X100. (197,198)

Quality control:

For Gram-positive: Staphylococcus aureus ATCC 25923

For Gram-negative: Escherichia coli ATCC 25922

2-14-2. Biochemical tests:

The following conventional methods were carried out for all Gram negative rods that were considered as suspected organisms:
2-14-2-1. Oxidase test:

Procedure:

Using a wooden stick, a colony of the test organism was removed and rubbed on the surface of a filter paper impregnated with oxidase reagent (Oxidase disk). (Appendix 2-B)

Within few seconds, the positive result was indicated by the development of a blue-purple colour whereas no colour change indicated a negative result. (198,199)

Quality control:

Positive control: *Pseudomonas aeruginosa*

Negative control: *Escherichia coli* ATCC 25922

2-14-2-2. Urease test:

Procedure:

The surface of Christensen’s urea slant (Appendix 1-H) was streaked with a portion of well isolated colony, then incubated at 37°C overnight.

A positive reaction was indicated by the change of the medium’s colour from pale yellow to purple pink. (197,200)

Quality control:

Positive control: *Proteus vulgaris*

Negative control: *Escherichia coli* ATCC 25922
2-14-2-3. Indole test:

Procedure:

A tube of peptone water (Appendix 1-I) was inoculated with the test organism. And Incubated at 37°C overnight. Drops of kovac’s reagent were added and shaked gently.

A positive reaction was indicated by a red ring at the surface of the medium while a colourless or yellow ring indicated a negative reaction. (196,198)

Quality control:

Positive control:  *Escherichia coli*  ATCC 25922

Negative control:  *Enterobacter aerogens*

2-14-2-4. Citrate utilization test:

Procedure:

The surface of Simmon’s medium (Appendix 1-J) was inoculated with straight wire, then incubated at 37°C overnight. A blue colour indicated positive reaction.

Original green colour and no growth indicated a negative reaction. (200)

Quality control:

Positive control:  *Klebsiella pneumoniae*

Negative control:  *Escherichia coli*  ATCC 25922
2-14-2-5. Motility test:

Procedure:

A tube of semi-solid motility medium (Appendix 1-K) was inoculated with straight wire by stabbing down the centre of the tube to about half the depth of the medium. Then the tube was incubated at 37°C overnight.

Motile bacterium gave diffuse spreading growth while non-motile bacteria gave growth that was confined to the stab line.\(^{(201,202)}\)

Quality control:

Positive control:  *Proteus mirabilis*

Negative control:  *Klebsiella pneumoniae*

2-14-2-6. Kligler iron agar:

Procedure:

A tube of kligler iron agar slope (Appendix 1-L) was inoculated with straight wire by stabbing the butt and streaking the slant of the medium and incubated at 37°C for 18 to 24 hours.

Reading of reactions as follows:

Red slant-red butt: no fermentation of glucose or lactose.

Red slant-yellow butt: glucose fermentation only.

Red slant-yellow butt and black precipitation: glucose fermentation +H\(_2\)S production.

Yellow slant-yellow butt: glucose and lactose fermentation.
The formation of gas was indicated by the presence of bubbles or cracks in the agar or by separation of the agar from the sides of the tube.\(^{(25,201)}\)

**Controls:**

<table>
<thead>
<tr>
<th></th>
<th>butt</th>
<th>Slant</th>
<th>Gas</th>
<th>(\text{H}_2\text{S})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>yellow</td>
<td>yellow</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 25922</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Red</td>
<td>Red</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>aeruginosa</em></td>
<td></td>
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</tr>
</tbody>
</table>

**2-14-2-7. Lysine decarboxylation test:**

**Procedure:**

A tube of lysine iron agar slope (Appendix 1-M) was inoculated with straight wire by stabbing the butt and streaking the slant of the medium, then incubated at 37°C overnight.

A positive lysine decarboxylation test was indicated by purple slant and butt while a negative reaction was indicated by a purple slant and yellow butt.

Hydrogen sulphide production was indicated by blackening in the medium.\(^{(25,200)}\)

**Quality control:**

**Positive control:** *Salmonella typhimurium* ATCC 14028

**Negative control:** *Shigella flexneri*
2-14-3. Analytical profile index (API) technique:

**a- Preparation of the strip:-**

An incubation box, tray and lid were prepared. About 5 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid chamber.

The strip was removed from its packaging and placed into the incubation box.

**b- Preparation of the inoculum:**

Sterile loop was used to remove a single well-isolated colony from the isolation plate and placed into a tube containing 5 ml sterile distilled water.

With the aid of sterile pipette, the colony was carefully emulsified to achieve a homogenous bacterial suspension.

**c- Inoculation of the strip:-**

Using the same pipette, both the tube and cupule were filled with the bacterial suspension for (CIT), (VP) and (GEL) tests.

For the other tests, only the tubes were filled with the bacterial suspension.

The tests ADH, LDC, ODC, H₂S and URE the tubes were covered with mineral oil to create anaerobiosis.

The incubation box was closed and incubated at 37°C for 18-24 hours.
Reading of the strip:-

After the incubation hours, the strip was read by referring to the reading table.

First the results of all spontaneous reactions were recorded on the report sheet. Then, reagents were added to VP, TDA and IND tests and their results were recorded. (Appendix 3-B)

Identification and interpretation:-

Using the identification table, the results recorded on the report sheet were compared with those given in the table.

The pattern of the reactions obtained was coded into numerical profile using the analytical profile index manual.

On the report sheet, the tests are separated into groups of 3 and a number 1, 2, or 4 was indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of the API 20 E strip. The oxidase reaction constituted the 21st test and had a value of 4 if it was positive. (203) (Appendix 3-A)

2-14-4. Serotyping of Shigella:-

Organisms identified as Shigella species were sub-cultured on nutrient agar plates. Using commercially anti-sera (Mast), serological tests were performed.
2-14-1. **Slide agglutination test for Shigella:-**

**Method:-**

1. Two to three drops of saline were added to a clean microscope slide.

2. Two to three colonies from the nutrient agar plate were picked using a disposable inoculation loop and emulsified with saline to form a homogeneous suspension.

3. A drop of Shigella polyvalent anti-serum was added and mixed by tilting the slide back and forth for 60 seconds.

The following Shigella polyvalent anti-sera were used:

- Polyvalent A for *Shigella dysenteriae*
- Polyvalent B for *Shigella flexneri*
- Polyvalent C for *Shigella boydii*
- Polyvalent D for *Shigella sonnei*

4. Distinct clumping or agglutination within the 60 seconds was regarded as positive results, and the Shigella was named according to the polyvalent anti-sera group used as *Shigella dysenteriae, Shigella flexneri, Shigella boydii* or *Shigella sonnei.*

2-14-5. **Identification and serotyping of diarrhoeagenic *E.coli* strains:-**

*E.coli* 1 and *E.coli* 2 were identified and given names according to their identification names by API 20 E system.
Other organisms identified as *E. coli* by API 20 E system were sub-cultured on sorbitol MacConkey agar, (Appendix 1-N) incubated at 37°C overnight and examined for growth. Those that appeared as colourless colonies were considered as non-sorbitol fermenters and were tested for agglutination with *E. coli* 0157 anti-serum (Difco). Those that were sorbitol fermenters (pink colonies) were sub-cultured on nutrient agar plates and serotyped by slide agglutination using diarrhaegenic *E. coli* anti-sera.

2-14-5-1. **Slide agglutination test for diarrhaegenic *E. coli*:**

**Method:**

A dense suspension of the organism was prepared by mixing 3-5 colonies in 3 ml saline in a small tube. The suspension was heated at 100°C for 60 minutes to destroy the heat-labile K-antigen and to allow the identification of the heat-stable O-antigen, and was then centrifuged at 900g. for 20 minutes.

The supernatant was removed, 0.5 ml saline was added to the precipitate and mixed well until a homogeneous suspension was achieved. This was used as antigenic suspension for O-antigen grouping.

Two drops of the antigenic suspension were placed on different sides of a microscope slide. A drop of polyvalent anti-serum (Mast) was added onto one of the drops and a drop of saline was added to the other as a control.

The reagents were mixed by tilting the slide back and forth for 60 seconds.
Distinct clumping or agglutination within this period, without clumping in the saline control (autoagglutination) was regarded as a positive result. Further slide agglutination tests were conducted as described above with monovalent anti-sera. (205)

2-14-6. Serotyping of Vibrios:-

Organisms identified as Vibrios were tested by slide agglutination using Vibrio 0-1 anti-sera. Those agglutinated were considered as cholera Vibrios. Non-agglutinated isolates were considered as non-cholera Vibrios. (206)

2-14-7. Identification of Campylobacter:-

2-14-7-1. Oxidase test:

**Procedure:**

Using a wooden stick, a colony of the test organism was removed and rubbed on the surface of a filter paper impregnated with oxidase reagent.

Within a few seconds, the positive result was indicated by the development of a blue-purple colour whereas no colour change indicated a negative result. (147,199)

**Quality control:**

**Positive control:** Pseudomonas aeruginosa

**Negative control:** Escherichia coli  ATCC 25922
2-14-7-2. Catalase test:

Procedure:

Using a wooden applicator stick, a colony of the test organism was removed and dipped into a small amount of 3% hydrogen peroxide solution in a clear small test tube.

Immediate rapid production of gas bubbles indicated a positive reaction. (197,198)

Quality control:

Positive control:  *Staphylococcus aureus* ATCC 25923

Negative control:  *Streptococcus pyogenes*

2-14-7-3. Motility test:

By simple wet mount method:

A drop of bacterial suspension was placed in the centre of a slide and covered with a cover glass.

The preparation was sealed with vaseline.

Motility was examined using 40x objective, closing sufficiently the condenser to give maximum contrast. (201)

Quality control:

Positive control:  *Proteus mirabilis*

Negative control:  *Klebsiella pneumoniae*
2-14-7-4. Hippurate hydrolysis test:

**Procedure:**

The organism under test was grown on a blood agar plate (Appendix 1-O) for 18 hours at 37°C in a microaerophilic atmosphere. A loopful of the colonial growth was suspended in 2 ml sterile distilled water, then 0.5 ml of 2% sodium hippurate solution (Appendix 2-C) was added and incubated at 37°C waterbath for 2 hours. One ml of ninhydrine solution (Appendix 2-D) was added and left up to 2 hours at room temperature. A positive reaction was indicated by the development of a purple colour\(^{147-201}\)

**Quality control:**

**Positive control:** *Streptococcus agalactiae*

**Negative control:** *Enterococcus faecalis*

2-14-7-5. Susceptibility to nalidixic acid and cephalothin:

**Procedure:**

1- A dense inoculums suspension of the organism was prepared in a tube of nutrient broth.

2- A sterile swab was soaked in this suspension, excess fluid was pressed out on the side of the tube, then the swab was rubbed over the surface of Columbia blood agar plate (Appendix 1-Q)

3- A 30µg nalidixic acid disk and 30µg cephalothin disk were applied.
4- The plate was incubated in a microaerophilic atmosphere at 37°C for 48 hours.

Sensitive organisms showed a clear zone of inhibition of growth around the disk, while resistant organisms grew up to the edge of the disk. (25,147)

2-14-8. Further identification tests for *Aeromonas hydrophila*:

2-14-8-1. Aesculin hydrolysis test:

**Procedure:**

Aesculin medium (Appendix 1-R) was inoculated and incubated overnight at 37°C. A positive reaction was indicated by blackening of the medium. (25,199)

**Quality control:**

**Positive control:** *Enterococcus faecalis*

**Negative control:** *Streptococcus pyogenes*

2-14-8-2. β-haemolysis on blood agar:

**Procedure:**

A well selected colony from MacConkey agar was taken by a wire loop and sub-cultured on a blood agar plate and incubated aerobically at 37°C for 16-18 hours.

*Aeromonas hydrophila* produced relatively large colonies surrounded by a zone of β-haemolysis. (14-24)
2-14-8-3. Susceptibility to ciprofloxacin and cephalothin:

Procedure:

A suspension of the organism was prepared in sterile distilled water in a small tube (equivalent to McFarland 0.5 turbidity standard). (Appendix 2-E)

Sterile cotton swab was dipped into the suspension and spread over the surface of the medium.

Ciprofloxacin and cephalothin disks were applied using sterile forceps and pressed gently to ensure even contact.

The plate was incubated at 37°C overnight.

*Aeromonas hydrophila* showed a large zone of inhibition around the ciprofloxacin disk, while no inhibition zone appeared around the cephalothin disk (14-24).

**Quality control:** *E.coli* ATCC 25922

2-14-9. Further identification tests for Morganella morgani:

For organisms identified as Morganella morgani the following additional tests were done:

2-14-9-1. Phenylalanine deaminase test:

Procedure:

A universal container with phenylalanine agar (Appendix 1-S) was inoculated and incubated at 37°C overnight. A few drops of 10% ferric chloride solution (Appendix 2-F) were added over the surface of the growth.
Development of green colour in the fluid and on the slope indicated a positive reaction. No green colour indicated a negative reaction. (200,207)

Quality control:

Positive control: *Proteus vulgaris*

Negative control: *E.coli* ATCC 25922

2-14-9-2. Swarming on agar medium:

A colony of the organism was sub-cultured on a nutrient agar plate (Appendix.1-T) in order to obtain single discrete colonies.

The plate was incubated at 37°C overnight.

Swarming organisms spread over the surface of the agar medium usually in a series of successive waves, while non-swarming organisms do not spread and appear as single discrete colonies. *Morganella morgani* does not swarm. (199,202)

Quality control:

*Proteus vulgaris*: swarming

*E.coli*: no swarming

2-15. Antimicrobial susceptibility testing:

The antimicrobial activity against six antimicrobials for all isolates was determined by Kirby-Bauer disk diffusion method, using Mueller Hinton agar (Appendix 1-U), and following standard CLSI technique.
The following antimicrobials were used:

Amoxy-clavulanic acid    AMC 10/20 mcg
Ciprofloxacin             CPR 5mcg
Ceftriaxone               CTR 30mcg
Cefixime                  CFM 5 mcg
Co-trimoxazole            COT 1.125/23.75 mcg
Gentamicin                CN 10mcg

**Procedure:**

1- Using a wire loop, a well-isolated colony was picked and transferred into a tube containing 5 ml normal saline.

2- The turbidity of the suspension was adjusted with 0.5 McFarland turbidity standard. (Appendix 2-E)

3- Sterile swab was dipped into the inoculum suspension and rotated several times with firm pressure on the inside wall of the tube to remove excess fluid.

4- The swab was used to inoculate a well-dried surface Mueller-Hinton agar plate by streaking the swab three times of the entire agar surface, rotating the plate in three directions.

The plate was kept on the bench for 3-5 minutes for the surface of the agar to become dry before adding the antimicrobial disks.
5- Using sterile forceps, the antimicrobial disks were placed on the surface of the agar plate. The disks were pressed gently on the agar surface to provide uniform contact. The disks were evenly distributed on the agar so that they are no closer than 24mm from center to center to avoid interference between zones of inhibition.

6- The plates were inverted and incubated at 35-37°C aerobically for 16-18 hours which is the standard time of incubation for measuring zone diameters.

7- Using a ruler, the zones of complete growth inhibition around each of the disks were carefully measured to within the nearest millimeter, the diameter of the disk is included in this measurement. The interpretative correlates (susceptible, intermediate or resistant) was produced by referring to CLSI published tables. (25-208).

Quality control:

*Staphylococcus aureus*  
*ATCC  25923*

*Escherichia coli*  
*ATCC  25922*
CHAPTER THREE

Results

3-1. Distribution of adults and children:

As illustrated in Fig (1), 550 (61.1%) stool specimens were collected from children and 350 (38.9%) from adults.

3-2. Macroscopic and microscopic examination:

As showed in Fig(2), the macroscopic and microscopic examination of the 900 stool specimens showed the following:-

RBCs, pus cells mucus were present in 290 specimens

Pus cells only were found in 150 specimens.

RBCs only were found in 80 specimens

Mucus only was found in 210 specimens

Abnormal components were absent in 170 specimens

3-3. Laboratory findings:

As illustrated in Fig(3), culture of 900 stool specimens, yielded 232 (25.77%) enteric pathogens, of which 158 (68.5%) were isolated from children, while 73 (31.5%) were from adults.

Fig(4) showed that diarrhaegenic *E.coli* strains were the most common isolates, seen 124 (53.45%), followed by Shigella spp. in 38 (16.38%) specimens, Salmonella spp. in 19 (8.19%), Campylobacter spp. were seen in 15 (6.47%), and also *Aeromonas hydrophila* were seen in 15 (6.47%)
specimens, *Morganella morgani* were found in 13 (5.60%) specimens, *Yersinia enterocolitica* were seen 5 (2.16%) and non-cholera Vibrios in 3 (1.29%) specimens.

As illustrated in Fig (5), out of the 124 DEC isolates, 97(78.2%) were isolated from children and only 27 (21.8%) from adults. Fig (6) showed that *Enteropathogenic E.coli* (EPEC) were the most frequent isolates, 31(25%) followed by *E.coli* (1) 22 (17.74), *E.coli* (2) 20 (16.13%), *Enterotoxigenic E.coli* (ETEC) 20 (16.13%) and *Enterohaemorrhagic E.coli* 10 (8%).

Fig (7) showed that most Shigellae 24 (63.1%) were isolated from children while 14 (36.9%) were isolated from adults.

As illustrated in Fig (8) *Shigella flexneri* was isolated from 30 patients (78.94%) followed by *Shigella dysenteriae* 5 (13.15%) and *Shigella boydii* 3 (7.98%).

Fig (9) showed that most Salmonella were isolated from adults 12 (63.1%) and only 7 (36.9%) were isolated from children.

Fig (10) showed that *Salmonella cholera,arizonae* was the most common non-typhoidal Salmonella isolated 6 (35.29%), followed by *Salmonella typhimurium* 2 (11.76%).

As illustrated in Fig (11) 9 (60%) of Campylobacter species were isolated from children and 6 (40%) were from adults.

Fig (12) showed that *Campylobacter jejuni* was the most frequent 7 (77.78%) of the three species of *Campylobacter* isolated, followed by *Campylobacter coli* 5 (20%) and *Campylobacter lari* 3 (30.3%).
3-4. Relationship between isolates and microscopic examination of stool specimens:

In comparison between the microscopic examination of stool specimens examined in this study and the organisms isolated, we found that all Shigella species, E.coli (1), E.coli (2), Campylobacter and Yersinia were isolated from stools containing RBCs, pus cells and mucus while EHEC strains were isolated from both stools that containing RBCs, pus cells and stools containing RBCs only. Other strains of diarrheagenic E.coli were isolated from stools with different microscopic appearance. Aeromonas and Vibrios were isolated only from watery stool that contained mucus. Non-typhoidal Salmonella were isolated from stools that contained mucus, stools that contain pus cells and also from stools without any abnormal components. Morganella was isolated from stools that contained pus cells only and from stools with mucus only.

3-5. Antimicrobial susceptibility testing:

As illustrated in Fig (13) and indicated in Table (1), 225 (96.98%) of all pathogens were susceptible to ciprofloxacin. Among all isolates 170 (73.28%) and 82 (35.34 %) were susceptible to gentamicin and ceftriaxone respectively. For amoxy-clavulanic acid only 4 (1.72%) strains were susceptible. All the 38 Shigella species were susceptible to ciprofloxacin, ceftriaxone and cefixime while 28 (73.68%) were susceptible to co-trimoxazole and only 4 (1.72%) were susceptible to amoxy-clavulanic acid.

Among DEC strains, 122 (98.39%) were susceptible to ciprofloxacin, 115 (92.74%) were susceptible to ceftriaxone, 110 (88.71%) were
susceptible to cefixime and 77 (62.10%) were susceptible to gentamicin. The susceptibility to co-trimoxazole was shown by 28 (22.58%) and to amoxy-clavulanic acid by only 2 (1.61%) 

All the isolated strains of Salmonella species 19 (100%) were susceptible to ciprofloxacin, ceftriaxone and cefixime 16 (84.21%) and 15 (78.95%) were susceptible to gentamicin and co-trimoxazole respectively. All strains were resistant to amoxy-clavulanic acid.

The 15 isolates of *Aeromonas hydrophila* were susceptible to ciprofloxacin, ceftriaxone, cefixime and co-trimoxazole while 12 (80%) isolates were susceptible to gentamicin. All isolates were resistant to amoxy-clavulanic acid.

All isolates of *Morganella morgani* were susceptible to ciprofloxacin, ceftriaxone, cefixime, and gentamicin. Only 7 (53.85) were susceptible to co-trimoxazole, while all isolates were resistant to amoxy-clavulanic acid.

All Campylobacter species were susceptible to gentamicin, 80% of the isolates were susceptible to erythromycin, (73.33%) were susceptible to doxycycline, while (66.67%) of the isolates were susceptible to both ciprofloxacin and nalidixic acid, 4 (26.67%) were susceptible to ceftriaxone. All isolates were resistant to amoxy-clavulanic acid, cefixime and co-trimoxazole.

The 5 strains of *Yersinia enterocolitica* isolated in this study showed 100% resistance to amoxy-clavulanic acid and gentamicin and 100% susceptibility to ciprofloxacin, ceftriaxone, and cefixime.
The 3 non-cholera Vibrios isolates showed 100% susceptibility to ciprofloxacin, ceftriaxone, and cefixime, and 100% resistance to amoxy-clavulanic acid and co-trimoxazole.
Fig. (1) Distribution of adults and children in the study group
**Fig (2):** Direct microscopic examination of stool specimens from the study sample
Fig (3): Frequency of pathogenic organisms isolated from adults and children of the study group
Fig (4): Frequency of pathogenic organisms isolated

- Diarrheogenic E. coli: 124
- Shigella species: 38
- Salmonella species: 19
- Campylobacter species: 15
- Aeromonas hydrophila: 15
- Morganella morganii: 13
- Yersinia enterocolitica: 5
- Non-cholera vibrios: 3

Organisms:
- Diarrheogenic E. coli: 53.5%
- Shigella species: 16.4%
- Salmonella species: 8.2%
- Campylobacter species: 6.5%
- Aeromonas hydrophila: 6.5%
- Morganella morganii: 5.6%
- Yersinia enterocolitica: 2.2%
- Non-cholera vibrios: 1.3%
Fig (5): Frequency of diarrhaegenic *E. coli* in adults and children
Fig (6): Serotyping distribution of diarrheagenic E. coli

- Enteropathogenic E. coli: 31 (25%)
- Entertoxigenic E. coli: 20 (16.1%)
- Enterohaemorrhagic E. coli: 10 (8.0%)
- Other strains: 21 (16.9%)
- E. coli[1]: 22 (17.7%)
- E. coli[2]: 20 (16.1%)
Fig (7): Frequency distribution of Shigella species in adults and children

24 (63.2%)
14 (36.8%)
Fig (8): Serotyping distribution of Shigella species
Fig (9): Frequency distribution of Salmonella species in adults and children

- Adults: 12 (63.1%)
- Children: 7 (36.9%)
Fig (10): Serotyping distribution of Salmonella species

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella cholera arizonae</td>
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<td>31.6%</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>2</td>
<td>10.5%</td>
</tr>
<tr>
<td>Salmonella paratyphi B</td>
<td>4</td>
<td>21.1%</td>
</tr>
<tr>
<td>Salmonella species</td>
<td>7</td>
<td>36.8%</td>
</tr>
</tbody>
</table>
Fig (11): Frequency distribution of Campylobacter species in adults and children
Fig (12): Biotyping distribution of Campylobacter species
Fig (13): Percentages of isolates susceptible to antimicrobials
Table (1): Antimicrobial susceptibility pattern of the pathogenic bacteria isolated

<table>
<thead>
<tr>
<th>Pathogen tested</th>
<th>AMC</th>
<th>CPR</th>
<th>CTR</th>
<th>CFM</th>
<th>COT</th>
<th>CN</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
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<tr>
<td>All isolates</td>
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<td>1.6</td>
<td>0.0</td>
<td>98.3</td>
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<td>100.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Non-cholera Vibrios</td>
<td>100.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**AMC**: amoxy-clavulanic acid  **CPR**: Ciprofloxacin  **CTR**: Ceftriaxone  **COT**: Co-trimoxazole  **CFM**: Cefixime  **CN**: Gentamicin

**R**: Resistant  **I**: Intermediate  **S**: Susceptible
Table (2): Colonial morphology of the isolates on the different types of media used

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Mac. No(3)</th>
<th>S.M</th>
<th>DCA</th>
<th>XLD</th>
<th>HE</th>
<th>TCBS</th>
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</thead>
<tbody>
<tr>
<td>Shigella dysenteriae</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Shigella boydii</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Salmonella cholera.arizonae</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Other salmonellae</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Diarrhoeagenic E.coli (most strains)</td>
<td>LF</td>
<td>SF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>NG</td>
</tr>
<tr>
<td>ETEC 0111</td>
<td>LF</td>
<td>SF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>NG</td>
</tr>
<tr>
<td>E.coli 0157</td>
<td>LF</td>
<td>NSF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>NG</td>
</tr>
<tr>
<td>E.coli (1)</td>
<td>NLF</td>
<td>SF</td>
<td>NG</td>
<td>LF</td>
<td>Su F</td>
<td>NG</td>
</tr>
<tr>
<td>E.coli (2)</td>
<td>NLF</td>
<td>NSF</td>
<td>NG</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>NLF</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Morganella morgani</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>Su F</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>Su F</td>
<td>NLF</td>
<td>NG</td>
</tr>
<tr>
<td>Non-cholera vibrios</td>
<td>NLF</td>
<td></td>
<td>NLF</td>
<td>Su F</td>
<td>NG</td>
<td>Su F</td>
</tr>
</tbody>
</table>

SM : sorbitol mac. agar  
HE : Hekten enteric agar  
LF : lactose fermenter  
SF : sorbitol fermenter  
Su F : sucrose fermenter  
NG : no growth  
NLF : non-lactose fermenter  
NSF : non-sorbitol fermenter

Colour of colonies :-
Sorbitol fermenter : pink  
Non-sorbitol fermenter : colorless  
Lactose fermenter on Mac. No.3 : pink  
Non-lactose fermenter on Mac. No.3 : colorless  
Lactose fermenter on XLD : yellow  
Non-lactose fermenter on XLD : pink  
Lactose fermenter on HE : pink to orange  
Non-lactose fermenter on HE : Blue – green
### Table 3: Biochemical reactions of the pathogenic organisms isolated

<table>
<thead>
<tr>
<th></th>
<th>Mac.</th>
<th>Oxidase</th>
<th>Motility</th>
<th>Urease</th>
<th>Citrate</th>
<th>Indole</th>
<th>LDC</th>
<th>Slope</th>
<th>Butt</th>
<th>His</th>
<th>gas</th>
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<tbody>
<tr>
<td><strong>Shigella dysenteriae</strong></td>
<td>NLF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Shigella flexneri</strong></td>
<td>NLF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Shigella boydii</strong></td>
<td>NLF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Salmonella cholera.arizonae</strong></td>
<td>NLF</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Red</td>
<td>yellow</td>
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<tr>
<td><strong>Salmonella typhimurium</strong></td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>Red</td>
<td>yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Other salmonellae</strong></td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Red</td>
<td>yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Diarrhoeagenic E.coli</strong> (most strains)**</td>
<td>LF</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>yellow</td>
<td>yellow</td>
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<td>+</td>
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<td><strong>ETEC 0111</strong></td>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>yellow</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
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<td>yellow</td>
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<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Aeromonas hydrophila</strong></td>
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<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Red</td>
<td>yellow</td>
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<td>-</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Yersinia enterocolitica</strong></td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Non-cholera vibrios</strong></td>
<td>NLF</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Red</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mac. : MacConkey agar  
LF : lactose fermenter  
LDC : lysine decarboxylation  
NLF : non lactose fermenter  

Kligler iron agar

App. 4- A  
App. 4- B  
App. 4- C  
App. 4- D  
App. 4- E  
App. 4- F  
App. 4- G  
App. 4- H  
App. 4- I  
App. 4- J  
App. 4- K  
App. 4- L
Table (4): Biochemical reactions of *Campylobacter* species isolated

<table>
<thead>
<tr>
<th></th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Motility</th>
<th>Hippurate hydrolysis</th>
<th>Susceptibility to</th>
<th>Nalidixic acid</th>
<th>Cefalothin</th>
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<tr>
<td><em>Campylobacter jejuni</em></td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>S</td>
<td>R</td>
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<tr>
<td><em>Campylobacter coli</em></td>
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<td>+</td>
<td>-</td>
<td>S</td>
<td>R</td>
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<tr>
<td><em>Campylobacter lari</em></td>
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<td>-</td>
<td>R</td>
<td>R</td>
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Table(5): Differentiation between *Aeromonas hydrophila* & non-cholera vibrios

<table>
<thead>
<tr>
<th></th>
<th>Growth on MacConkey agar</th>
<th>Growth on TCBS</th>
<th>β-haemolysis on Blood agar</th>
<th>Aesculin hydrolysis</th>
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<td>+</td>
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<tr>
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<td>NLF</td>
<td>Yellow colonies</td>
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<td>-</td>
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</tbody>
</table>
Table (6): Differentiation between *Morganella*, *Providencia* & *Proteus* spp.

<table>
<thead>
<tr>
<th></th>
<th>Mac.</th>
<th>Swarming</th>
<th>Urease</th>
<th>Citrate</th>
<th>Indole</th>
<th>H2S</th>
<th>Motility</th>
<th>PDA</th>
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<td>-</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Providencia spp.</em></td>
<td>NLF</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Proteus spp.</em></td>
<td>NLF</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Mac. : MaConkey agar  
H2S : Hydrogen sulphide  
PDA : Phenylalanine deamination
CHAPTER FOUR

: Discussion, Conclusion and Recommendations

4-1. Discussion

Diarrheal diseases remain a serious public health problem and continue to be a leading cause of death in children worldwide. (7,8)

In the present study, cultural techniques, standard biochemical and serological tests were used for isolation and identification of the bacterial etiological agents of diarrhea from stool specimens.

Potential enteropathogens were detected in 232 (25.8%) of the study group. It is in agreement with the previous study in Sudan by Waleed (2008) in which enteropathogens were detected in 26.4% of stool specimens. (85) Also it is quite similar to that reported by Medeiros from Brazil (2001) who isolated (22.8%) enteropathogens from 1836 episodes. (22)

The percentage of enteropathogens isolated in the current study is less compared with that reported in Tehran (2008) by Jafari who reported 45% enteropathogens isolated from 808 stool specimens. (84) This may be due to an improvement in isolation and identification techniques or due some local factors in this country.

Diarrheagenic \textit{E.coli} strains DEC are important pathogens that cause diarrheal diseases in many developing countries including Sudan. (26,27,85)

In the current study, the frequency of DEC was (13.8%) among patients in the study group and (53.4%) among enteropathogens isolated. This
may be due to the tendency of these organisms to cause different types of diarrhea in children and adults.\textsuperscript{(25,28)}

In the present study EPEC was found to be the most common strain among all DEC isolates, which represent 25%. The finding is quite similar to that reported by Eizo in Myanmar (2008) who detected 25.5% EPEC in his study.\textsuperscript{(59)}

The frequency of ETEC strains isolated in the current study was (16.1%) which is in agreement with that detected in Burkina Faso (2011) by Bonicaygon who reported (12.9%) ETEC strains isolated from 658 stool samples.\textsuperscript{(65)}

In the present study, the prevalence of EHEC was (8.0%) among the DEC isolates, which is close to the conducted study done in Tunisia by Al-Gallas (2007) in which (10.4%) EHEC isolates were reported.\textsuperscript{(7)} The isolation rate of EHEC in this study is considered as a high rate for this dangerous organism which needs more attention when examining stool specimens, especially that from children, although this organism can be easily isolated even in laboratories with low facilities.

Identification of most DEC strains requires many procedures and tests that include biochemicals, serological and modern molecular biological tests. Many of these tests are expensive and may not be available in most laboratories in Sudan. Only EHEC strains can be easily isolated and identified even in laboratories with low facilities.

Shigella species are important pathogens that causes bloody diarrhea in developing countries.\textsuperscript{(73,74,78,85)}
In the current study, the frequency of Shigella spp. was (4.2%) among patients in the study group and (16.4%) among enteropathogens isolated. The study is close to many studies conducted in Sudan and other countries. Zafar (2005) from Pakistan, detected (4.1%) Shigella spp. in his study, while the percentage of Shigella species within enteropathogens isolated was (16.0%) and (16.8%) as reported by Musa (2002) and Moez (2003) respectively. (80,78)

In this study, *Shigella flexneri* was dominant among all Shigella species isolated (78.9%). This is in agreement with previous studies conducted in Sudan and other African countries. In Sudan (77.0%) of *Shigella flexneri* was reported by Walid (2008) while (90%) was reported in Tanzania (2007) by Tema. (85,83)

Non-typhoidal Salmonella are one of the key aetiological agents of diarrheal diseases and food-borne infections in developed and developing countries. (88,108,181)

The current study identified 19 Salmonella species (8.19%) in the study group which is consistent with that reported in Singapore (1992) by Kamarasighe (10.8%) and (10.3%) reported in Bahrain by Ismaell (2002). (135,100) This isolation rate can be considered less in comparison with the isolation rate reported by Rollo (2004) which is (24.7%) and (22.68%) reported by Taneija (2002) from India. (102,101) These rates can be considered high in comparison with a previous study conducted in Sudan by El Safi, (2009), who isolated 3.4% Salmonella in his study. The same rates can be considered low in comparison with the 74.7% isolates
in Italy by Rollo (2004), and the 72.7% isolates in India (2002) by Taneja.

These rates reported in Sudan may be not a true reflection due to the nature of most Sudanese patients who do not usually seek medical attention or investigate their stools when suffering from diarrhea.

In this study 14 strains of non-typhoidal Salmonella are isolated from adults in comparison with 5 strains isolated from children. This may be due to consumption of some types of food like meat and chickens by adults and not by children, as most of NTS infections usually contracted through consumption of such types of food.

Campylobacter is recognized as major cause of gastroenteritis. The rate of Campylobacter infections worldwide has been increasing with the number of cases often exceeding those of salmonellosis and shigellosis. (110,120)

The frequency of Campylobacter isolates in the present study was (6.47%). This result is consistent with that reported in the literature by many researchers, (4.5%) by Zaman (1992) in Saudi Arabia, also (4.5%) by Ibrahim in Sudan (2000) and (8.3%) by Ugsal in Turkey (1997). (118,125,121) The slight increase in the isolation rate of Campylobacter in this study (6.6%) in comparison with previous studies in Sudan (4 to 4.5%), may be due to the proper collection and transportation of stool specimens and due to the improvement in the identification techniques.
Aeromonas species are increasingly recognized as enteric pathogens associated with acute gastroenteritis and traveler’s diarrhea. They represent serious public health concerns in many countries.\(^{(127,128,129)}\)

In the current study the rate of Aeromonas isolates was (6.47%), a result which is similar to those reported from other places; Maltouzoa from Greece (2001) reported (6.8%) as isolation rate of Aeromonas in his study while Bravo (2012) in Cuba reported (7.15%).\(^{(140,128)}\) These rates are less than the (12.1%) that was reported in Bangladesh by Hossain (1992).\(^{(136)}\) The decrease in the isolation rate of Aeromonas reported in some recent studies may be due to improvement in the water sanitation process as Aeromonas is considered an aquatic organism.\(^{(25,109)}\)

This may be the first study in which *Aeromonas hydrophila* was reported as a causative agent of diarrheal disease in Sudan.

*Yersinia enterocolitica* is identified as a cause of gastroenteritis in infants and young children and should be considered in cases of bacillary dysentery and enterocolitis.\(^{(141,142,144)}\)

The incidence rate of *Yersinia enterocolitica* reported in this study was (2.16%). This rate falls within the incidence rates reported in (1996) by Vaida (3.08%) and in (2003) by Andualen (1.5%).\(^{(152,153)}\)

This may be the first study to show isolation of *Yersinia enterocolitica* from stool of Sudanese patients, as this organism is known to be predominant in Europe and other countries of cold climate.\(^{(142,143)}\)
*Morganella morgani* has been reported as causing diarrhea in infants in the absence of other known bacterial enteropathogens, but its pathogenesis is not fully understood. \(^{(147,154,69)}\)

In this study all strains of *Morganella morgani* were isolated from children with diarrhea in the absence of any other bacterial pathogens. These isolates gave predominant growth of non-lactose fermenting colonies on MacConkey agar No.3 and DCA. This result is consistent with that reported by Hans who indicated that *Morganella morgani* have a significant role in diarrheal diseases. \(^{(158)}\)

In the present study, the frequency of *Morganella morgani* was (5.6%) among enteropathogens isolated. The finding is quite similar to that reported by Mahren (1990) who detected (6.38%) *M. morgani* in his study. \(^{(159)}\) A low frequency of *M. morgani* (1.37%) was detected by Hans (1986). \(^{(158)}\)

This may be the first report documenting the isolation of *M. morgani* from stools of Sudanese children.

Most literature recorded infections caused by *Vibrio* 01 and *Vibrio* 0139. Few literature about infections caused by non-01 *Vibrios*, although these infections are associated with diarrhea and some extraintestinal diseases. \(^{(87,161,162)}\)

In the present study, no *Vibrio* 01 or *Vibrio* 0139 was isolated. About 1.29% isolates of non-01 *Vibrios* was reported. These low isolation rates are consistent with that reported in the literature in which the isolation rate in Argentina (1996) by Notario was (0.35%) while in Uruguay
(2001) by Torres was (0.4%).\textsuperscript{(168,169)} The isolation rate is consistent with the 0.35\% reported in Argentina (1996) and the 0.4\% reported in Uruguay that NCV are not common causes of diarrheal disease in comparison with \textit{Vibrio cholerae} 01 and 0139 that can cause cholera epidemics.\textsuperscript{(162,163)}

Antimicrobial resistance is now recognized as an increasingly global problem.\textsuperscript{(175,176)}

In the current study, the level of antimicrobial resistance shown by DEC to amoxy-clavulanic acid, ciprofloxacin, ceftriaxone, cefixime, co-trimoxazole, and gentamicin were: (93.55\%), (1.61\%), (3.23\%), (8.87\%), (77.42\%) and (16.94\%) respectively. This high rate of resistance of DEC to amoxy-calavulanic acid and co-trimoxazole has been reported by many investigators from different countries.\textsuperscript{(58,63)}

In the present study all \textit{Shigella} species isolated showed a 100\% susceptibility to ciprofloxacin, ceftriaxone and cefixime with resistance rates 68.22\%, 76.32\% and 10.53\% to co-trimoxazole, amoxy-clavulanic acid and gentamicin respectively. This finding is consistent with many reports in the literature, with few reports about resistance of some strains of \textit{Shigella dysenteriae} to ciprofloxacin and other fluorinated quinlones.\textsuperscript{(179,182)}

The results obtained from this study can suggest the use of ciprofloxacin and the third generations cephalosporins as empirical drugs for treating shigellosis, especially at hospitals and health centers lacking good laboratory services.
In this study Salmonella species isolated showed almost the same pattern of antimicrobial susceptibility that given by Shigella. Ciprofloxacin and third generations cephalosporin also can be used for treating Salmonella infections with caution that some strains of Salmonella can be resistant to cefixime, cephoxtin and ceftriaxone due to the production of ESBLs,\(^{(183)}\) and the development of multidrug-resistant strains of non-typhoidal Salmonella among African children.\(^{(94)}\)

The present study showed a change in the antimicrobial susceptibility pattern of *Campylobacter* isolates due to the development of resistance to doxycycline, nalidixic acid and erythromycin, a new pattern of susceptibility that has not been reported in any previous study in Sudan. Also Campylobacters are the only group of organisms in this study that were 100% resistant to co-trimoxazole and cefixime, while *Yersinia enterocolitica* was the only organism that was 100% resistant to gentamicin. High resistance rates to amoxy-clavulanic acid and co-trimoxazole were common among most pathogens, although these drugs are widely used in Sudan for treating different types of infections, including respiratory and gastrointestinal tract infections. This resistance may be due to the development of resistant mutants or due to β-lactamases against β-lactam drugs.

There is difference in selective criteria of enteropathogens from stool specimens.

In the current study, 7 different types of selective and differential media were used. These include:-
DCA, XLD, HE, TCBS, MacConkey agar No.3, Skirrow and sorbitol MacConkey agar.

In this study we used DCA medium Leifson’s modification which contains less concentration of sodium desoxycholate (2.5 gm/L) in comparison with Hyne’s modification that contain 5 gram of sodium desoxycholate per litre. This low concentration of the bile salt make it less inhibitory for *Shigella dysenteriae* and *Salmonella paratyphi A.* (148,195)

Hektoen enteric agar (HE) was used in this study because it is a good differential and selective medium for isolation of Shigella and Salmonella species from enteric pathological specimens. It is often preferable when Shigellas are being sought as well as Salmonellae. It is more inhibitory to coliform and Proteus species but less inhibitory for Shigella because it contains high concentration of bile salt No.(3) (9 gram per litre), but its higher peptone contents (12 gram per litre) offsets the inhibitory effect of these bile salts on Shigella species. The use of double indicator (acid fuchsin + bromothymol blue) in this medium provides low toxicity effect and so improves recovery of pathogens. Three carbohydrates (lactose, sucrose and salicin) were incorporated in this medium. This gives better differentiation than lactose alone because some organisms that may be confused with pathogen (e.g. *Proteus* spp.) are non-lactose fermenters but they are sucrose fermenters. Salicin is a carbohydrate almost fermented by most Enterobacteriaceae except *Salmonella* and *Shigella* and this character helps in differentiation between organisms. This medium also contains high concentration of
lactose (12.0 grams per litre) which helps early recognition of slow lactose-fermenting organisms. XLD is a good selective and differential medium for the isolation of Salmonellae and Shigellae from clinical specimens. It has a good differentiation characters depending upon lactose, sucrose, xylose fermentation and lysine decarboxylation, but it is less selective for coliform and Proteus spp. because it contain low concentration of sodium deoxycholate (1 gram per litre) in comparison with DCA and HE media. Xylose which is an important component of this medium is known to be rapidly fermented almost by most Enterobacteriaceae except for the members of Shigella, Providencia and Edwardsiella. Shigella can be identified by its negative reactions on lactose, sucrose and lysine. Salmonella spp. are differentiated from non-pathogenic xylose fermenters by incorporation of lysine in the medium. Salmonella exhaust the xylose and decarboxylate the lysine, thus altering the pH to alkaline and mimicking the Shigella reaction. The high acid level produced by fermentation of lactose and sucrose prevents lysine-positive coliform from reverting the pH to an alkaline value, and non-pathogenic hydrogen sulphide producers do not decarboxylate lysine.

MacConkey agar No.3 was used as a differential and selective medium for diarrheogenic strains of *E.coli* as well as for Aeromonas, Morganella and Vibrios. It contains specially prepared fraction of bile salts (bile salt No.3) in addition to crystal violet. It becomes more selective than other formulations of MacConkey media and so gram positive cocci include staphylococci, micrococci and enterococci were completely inhibited. As
it contain lactose and neutral red indicator, it gave improved
differentiation between coliform and non-lactose fermenting organisms.
Also this medium is suitable for isolation of Salmonella and Shigella
from stool specimens.\textsuperscript{(148)} Appendix (1-E) Table (2)

All diarrheagenic strains of \textit{E.coli} (DEC) isolated in this study grew on
MacConkey agar No.3 as lactose fermenting (pink colonies) except the
\textit{E.coli} 1 and \textit{E.coli} 2 strains. They grew producing non-lactose
fermenting (colourless colonies) although these strains were considered
as highly pathogenic organisms.\textsuperscript{(193)} Table (2)

Sorbitol MacConkey agar is used as selective and differential medium for
the detection of \textit{E.coli} 0157. The formulation is identical to MacConkey
agar No.3 except that lactose has been replaced with sorbitol. \textit{Escherichia
coli} 0157 does not ferment sorbitol and therefore produces colourless
colonies. This medium is recommended as a simple, inexpensive, rapid
and reliable for screening \textit{E.coli} 0157.\textsuperscript{(43,44)} Appendix (1-N)

TCBS is used as a highly selective medium for pathogenic Vibrios due to
its high contents of bile salts (9 gms/l) and its alkaline PH (8.2). It
contain. sucrose and bromothymol blue to differentiate between sucrose
and non-sucrose fermenting Vibrios. In this medium most of the
enterobacteriaceae encountered in faeces are totally suppressed.\textsuperscript{(148,195)}

An important precaution must be considered when using this medium
that yellow colonies taken from it will give unsatisfactory oxidase
reaction, mainly a false negative due to the inhibitory to oxidase enzyme
to work well under acid conditions resulting from sucrose fermentation. Appendix (1-F).

Campylobacters were isolated using Skirrow selective medium which consist of Columbia agar base to which 5% lysed horse blood and Campylobacter selective supplement (Skirrow formulation) were added. This selective supplement contains vancomycin to inhibit growth of gram positive organisms, polymyxin B to inhibit gram negative organisms and trimethoprim to stop swarming of Proteus spp. as Proteus species can grow on most types of selective media used for stool culture.

In which the swarming is prevented by the action of bile salts which is a major component in these media. Skirrow medium does not contain bile salts so it needs addition of trimethoprem to stop this phenomenon. \(^{(148)}\) Appendix (1-F)

All Salmonella species isolated grew and gave clear colonial morphological appearance as non-lactose fermenting colonies on DCA, XLD and HE. Also, all Shigella species grew on the same types of media, except two strains of *Shigella dysenteriae*. Those two strains were identified from DCA and HE, not from XLD. They may be covered by lactose-fermenting organisms and other commensals due to the heavy inoculums used for culturing stool specimens as XLD is known to be a medium of moderate selectivity in comparison with DCA and HE.\(^{(148)}\)

The 5 isolates of *Yersinia enterocolitica* were identified from DCA and MacConkey agar because it is difficult to be identified from XLD as *Yersinia* produced yellow colonics on this medium due to its ability to
ferment sucrose. Coliform and other lactose fermenting organisms also produce yellow colonies when present on XLD.\textsuperscript{(148,195)} Table (2)

The results obtained from this study may suggest that always more than one selective media should be used and not to use XLD alone when culturing stool specimens.

All organisms isolated in this study, except that from the Vibrios group failed to grow on TCBS medium. As the isolation rate of Vibrios in this study was low 1.29% and these organisms can grow on MacConkey agar No.3 as well as on TCBS, we can suggest that it is not necessary to incorporate TCBS within other types of media used for routine examination of stools specimens unless requested. Table (2)

All Campylobacter species were isolated and identified from Skirrow medium that had been incubated under microaerophilic conditions. These conditions were achieved by two methods: anaerobic jar with microaerophilic gas generating kits and candle jar which is a practice non-expensive method. All Campylobacters isolates grew well in both methods of incubation.

The results obtained from this study can suggest that plates of Skirrow medium can be incubated in a candle jar in the absence of microaerophilic gas-generating kits.

In this study we used API 20E system for identification of Enterobactericeae and other related organisms like Vibrios and Aeromonas. API 20E is a good and specific identification system but it is
relatively expensive so it is not widely used in many hospitals and routine laboratories in Sudan.

In addition to (API 20E) identification system, we used some routinely conventional biochemical tests that include: oxidase, catalase, urease, citrate, indole and kligler iron agar. Table (14)

The results obtained from these tests gave a good approach for identification and help to exclude some organisms that were considered as non-pathogens when isolated from stool specimens like *Pseudomonas* and *Proteus*.

Also in this study we added some other single and economical tests that gave a good preliminary and sometimes tentative identification of some organisms. This may be useful in health centres lacking good bacteriological services. These tests include lysine decarboxylation, phenylalanine, deamination, aesculin hydrolysis, haemolysis on blood agar, inability to swarm on nutrient agar and susceptibility to cephalothin and ciprofloxacin.

Lysine decarboxylation test improved identification of *Salmonella* and differentiate it from *Shigella*, because *Salmonellae* are the only recognized group of Enterobacteriaceae which regularly decarboxylate lysine rapidly and which produce large amounts of hydrogen sulphide. (25,200)

The positive phenylalanine test plus its inability to swarm on nutrient agar medium, improved identification of *Morganella morgani*, as *Morganella morgani*, Proteus, and Providencia are known to be the only
group of Enterobactericeae that can deaminate phenylalanine. Further
tests were used in this study to differentiate Morganella from these
organisms.\textsuperscript{200,207} Table (6)

Aesculin hydrolysis test, plus production of β-haemolysis on blood agar
and susceptibility to cephalothin and ciprofloxacin improved
identification of Aeromonas. Aeromonas is known to be the only oxidase-
positive Gram-negative bacilli that can hydrolize aesculin and be naturally
resistant to ampicillin and first generations of cephalosporins.\textsuperscript{14,24}

This may be due to the ability of Aeromonas to express chromosomal β-
lactam induced β-lactamases that can not be detected by conventional
susceptibility methods.

Production of colonies surrounded by a zone of β-haemolysis by
\textit{Aeromonas hydrophila} is a useful character that can help in its
identification.

Most of the enteropathogens reported in this study were Gram-negative
rods, non-lactose fermenters (except most strains of \textit{E. coli}), oxidase-
negative with few exceptions and urease-negative also with few
exceptions.

Many investigators in Sudan, when culturing stool specimens, usually
considere oxidase-positive organisms as Pseudomonas species, while this
reaction can be given also by Aeromonas and Vibrios. Also some
investigators consider urease-positive organisms as Proteus species while
Yersinia and Morganella are also urease-positive.
The results obtained in the present study can suggest that more biochemical tests should be performed for all non-lactose fermenting organisms isolated from stool specimens before deciding that they are non-pathogens.

4-2. Conclusion

The current study provided important knowledge about isolation, identification and antimicrobial susceptibility pattern of aerobic bacteria that cause diarrheal diseases.

In this study 8 genera and more than 10 species of enteric organisms were isolated.

Diarrhaegenic strains of *E. coli* and *Shigella* species were the most common isolates reported in this study.

Enterohaemorrhagic *E. coli* 0157, which cause a type of diarrhea that may lead to life-threatening diseases, can be easily isolated and identified, even in laboratories with low facilities.

The relatively high isolation rate of *Aeromonas* and *Morganella* species in this study (>5%), can indicate their role in diarrheal diseases.

The use of different combinations of media with varying degree of selectivity can increase the chance of isolation of suspected bacterial pathogens from stool specimens.

The use of conventional biochemical tests that are routinely used for identification of Gram-negative bacilli, can play an important role in the identification of many pathogens isolated from stool specimens.
especially in laboratories lacking modern identification techniques. Also, the results of these tests can act as an inclusion criteria for organisms suspected to be causative agents of diarrhea and may need additional identification tests. Also, these tests can act as an exclusion criteria for organisms considered commensals of human gastro intestinal tract like Proteus and Pseudomonas which can be excluded without performing other expensive tests.

The use of a transport medium like Cary-Blair is an essential step for isolation of Campylobacter species from stool specimens.

Candle jar, can be used instead of Campylobacter gas generating kits (which are too expensive) as a method for achieving microaerophilic conditions when incubating cultures in which we suspect to isolate Campylobacter species.

Antimicrobial susceptibility pattern of the isolates showed that ciprofloxacin and ceftriaxone were the most effective drugs for infectious diarrhea while amoxy-clavulanic acid is the least effective one. Other drugs like co-trimoxazole, cefixime and gentamicin can be used depending on the result of their susceptibility test against the isolated organism.
4-3. Recommendations

To use different combinations of selective media including HE and MacConkey No.3 in order to provide more opportunities for the recovery of enteric pathogens for stool specimens.

All stool cultures from patients with bloody diarrhea, children, should be examined for Enterohaemorrhagic *E. coli* 0157 (EHEC).

More studies should be continued to determine the pathogenesis and role of *Morganella morgani* in diarrheal diseases of children and the role of *Aeromonas hydrophila* as a causative agent of water-borne diseases.

To make the laboratory investigations cost-effective, conventional biochemical tests should be used before serological tests, and other expensive techniques.

Rapid diagnostic methods should be included to decrease the burden of diarrheal diseases especially among children infected with diarrhaegenic strains of *E. coli* and Shigella species.

Ciprofloxacin, ceftriaxone and gentamicin should be considered as drugs of choice in cases of diarrhea in need of treatment.

In order to prevent infectious diarrheal diseases and their consequences, currently available vaccines against enteric pathogens should be used and the development of new active vaccines against the most important agents of infectious diarrhea is advised.
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153- Andualem, B. and Geyid, A. (2003). The prevalence of Yersinia enterocolitica isolates in comparison to those of the commonly


Appendices

Appendices (1) Culture Media

Appendices (2) Reagents

Appendices (3) API 20E

Appendices (4) Identification tests

Appendices (5) Tables

Appendices (6) Questionnaire

Appendices (1)

Appendix (1-A):

Cary-Blair Medium LAB M LAB 505

Formula: g/litre
Sodium hydrogen phosphate 1.1
Sodium thioglycollate 1.5
Sodium chloride 5.0
Calcium chloride 0.09
Agar 5.6

Direction of preparation: 13.3 grams of powder was suspended in a litre of distilled water. The contents was boiled to dissolve completely, then dispersed into small screw caped bottles. The medium was sterilized by free streaming in an autoclave for 10 minutes. Then the medium was cooled and tightened caps.

Positive control: Shigella dysenteriae

Negative control: uninoculated medium
Appendix (1-B):

**Desoxycholate Citrate Agar** - LAB M - LAB 29

**Formula:**
- Beef extract: 5.0 g/L
- Balanced peptone No. 1: 5.0 g/L
- Lactose: 10.40 g/L
- Sodium citrate: 5.0 g/L
- Sodium thiosulphate: 5.0 g/L
- Ferric citrate: 1.0 g/L
- Sodium desoxycholate: 2.0 g/L
- Neutral red: 0.025 g/L
- Agar No. 2: 12.0 g/L

45.5 grams of powder was dispersed in 1 litre of distilled water, then soaked for 10 minutes, the medium was sterilized by boiling, cooled at 45°C, mixed then poured into sterile Petri dishes.

**Positive control:** *Salmonella typhimurium* ATCC 14028

**Negative control:** *Enterococcus faecalis*

Appendix (1-C):

**XLD agar** - Conda 1080.00

**Formula:**
- Lactose monohydrate: 7.5 g/litre
- Sucrose: 7.5 g/litre
- Sodium thiosulphate: 6.8 g/litre
- L.lysine: 5.0 g/litre
- Sodium chloride: 5.0 g/litre
- Xylose: 3.5 g/litre
- Yeast extract: 3.0 g/litre
- Sodium desoxycholate: 2.5 g/litre
- Ferric ammonium citrate: 0.8 g/litre
Phenol red 0.08
Bacteriological agar 13.5
55.2 grams of powder was dispersed in one litre of distilled water, then heated until the medium was boiled, cooled to 50˚c, and poured into Petri dishes.
Positive control: *Salmonella typhimurium* ATCC 14028

Negative control: *E.coli* ATCC 25923

Appendix (1-D):

**Hektoen Enteric Agar** Mast diagnostic Dm 134 D

**Formula:**

- Peptone 25.0
- Lactose 10.0
- Sucrose 12.0
- Salicin 1.0
- Sodium chloride 2.0
- Sodium thiosulphate 1.0
- Ferric ammonium citrate 2.0
- Trisodium citrate 1.25
- Bile salt 1.5
- Acid fuchsin 0.025
- Bromothymole blue 0.05
- Agar A 14.0

**Directions:**
69.8 grams of powder was dispersed in 1 litre of distilled water, heated gently and boiled for a few minutes, cooled to 50˚c, then poured into sterile Petri dishes.

Positive controls: *Salmonella typhimurium* ATCC 14028

*Shigella flexneri*

Negative control: *Escherichia coli* ATCC 25922

Appendix (1-E):
MacConkey Agar No.3  Code: CM115

**Formula:**

<table>
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<th>Component</th>
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<td>Lactose</td>
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<td>Bile salts No.3</td>
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<td>Crystal violet</td>
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<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH 7.3 ± 0.2</td>
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</table>

**Directions:**

51.5 grams of powder was dispersed in 1 litre of distilled water, boiled until dissolved completely, then the medium was sterilized by autoclaving at 121°C for 15 minutes, cooled and poured into sterile Petri dishes.

**Positive control:**  *Salmonella typhimurium* ATCC 14028

**Negative control:**  *E.coli* ATCC 25923

**Appendix (1-F):**

**T.C.B.S Cholera Medium**  LAB  M  LAB 96

**Formula:**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/litre</th>
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</thead>
<tbody>
<tr>
<td>Yeast extract</td>
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<tr>
<td>Peptone mix</td>
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</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>10.0</td>
</tr>
<tr>
<td>Bile salts</td>
<td>9.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10.0</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>1.0</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.04</td>
</tr>
<tr>
<td>Thymol blue</td>
<td>0.04</td>
</tr>
<tr>
<td>Agar No.1</td>
<td>15.0</td>
</tr>
</tbody>
</table>
**Directions:**

88 grams of powder was dispersed in 1 litre of distilled water, mixed well and sterilized by boiling for few minutes, cooled to 50°C, then poured into sterile Petri dishes

**Positive control:** Non-cholera vibrio

**Negative control:** *E.coli* ATCC 25922

**Appendix (1-G):**

**Cambylobacter selective medium (Skirrow)**

Selective supplement code SR 69

Vial contents: (each vial is sufficient for 500ml of medium)

- Vancomycin 5 mg
- Polymyxin 1.2501 U
- Trimethoprim 2.5 mg

Directions:-

The contents of the vial was rehydrated by adding two ml of distilled water and turn end-over-end to dissolve and added to 500 ml of sterile Columbia agar base cooled to 50-55°C. Then 5% lysed horse blood was added mixed gently and poured into sterile petri dishes.

**Positive control:** *Campylobacter jejuni* ATCC 29428

**Negative control:** *Escherichia coli* ATCC 25922
Appendix (1-H):

Urea agar base Oxoid CM 53

Formula:
- Peptone 1.0  
- Glucose 1.0  
- Sodium chloride 5.0  
- Disodium phosphate 1.2  
- Potassium dihydrogen phosphate 0.8  
- Phenol red 0.012  
- Agar 15.0

Directions:
2.4 grams of urea agar base was suspended in 95ml of distilled water, heated gently until dissolved completely, sterilized by autoclaving at 115°C for 20 minutes, cooled to 50°C, then 5ml of sterile 40% Urea solution was added mixed well and distributed in sterile small tubes and allow to set in the slope position.

Appendix (1-I):

Peptone water Oxoid Code:CM9

Formula:-
- Peptone 10.0  
- Sodium chloride 5.0  
- pH 7.2 ± 0.2

Directions
15 grams of medium was dissolved in 1 litre of distilled water, mixed well and distributed into small containers, the medium was sterilized by autoclaving at 121°C for 15 minutes.
Appendix (1-J):

Simmons citrate agar  Oxoid  CM 155

**Formula:**
- Magnesium sulphate  0.2
- Ammonium dihydrogen phosphate  0.2
- Sodium ammonium phosphate  0.8
- Sodium citrate  2.0
- Sodium chloride  5.0
- Bromothymol blue  0.08

**Directions:**
23 grams of medium was suspended in 1 litre of distilled water and boiled until dissolved completely, the medium was distributed into small test tubes and sterilized by autoclaving at 121˚c for 15 minutes. After sterilization the tubes were allowed to set in slope position.

Appendix (1-K):

Motility test medium  Himedia  M 260

**Formula:**
- Tryptone  10.0
- Sodium chloride  5.0
- Agar  5.0

Twenty grams of powder was suspended in 1 litre of distilled water, then boiled until dissolved completely, then the medium was dispenced in small tubes and sterilized by autoclaving at 121˚c for 15 minutes. After sterilization the tubes were allowed to cool in upright position.
Appendix (1-L):

**Kilgler iron agar:** Oxoid CM 33

**Formula:** gm/litre

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab-lemco powder</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>20.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.3</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.05</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
</tbody>
</table>

**Directions:**

Fifty five grams of powder was suspended in 1 litre of distilled water, heated until dissolved completely, the medium was distributed into test tubes and sterilized by autoclaving at 121°C for 15 minutes. Then allowed to set as slope with 1 in butts.

Appendix (1-M):

**Lysine Iron Agar** LAB M LAB 54

**Formula:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced peptone No. 1</td>
<td>5.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
</tr>
<tr>
<td>L-lysine</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.04</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>0.02</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
</tbody>
</table>
**Directions:**
31.5 grams of powder was dispersed into 1 litre of distilled water, dissolved completely by boiling, then distributed into tubes and sterilized by autoclaving at 121˚c for 15minutes. After sterilization the tubes were allowed to set in slope position.

**Controls:** *Enterobacter aerogenes – Salmonella typhimurium* :

- Lysine decarboxylation

*Proteus mirabilis:* deamination

**Negative control:** *Shigella flexneri*

**Appendix (1-N):**

**Sorbitol MacConkey Agar**  LAB M   LAB 161

**Formula:** grams/litre
- Peptone  20.0
- Sorbitol  10.0
- Bile salts No. 3  1.5
- Sodium chloride  5.0
- Neutral red  0.03
- Crystal violet  0.001
- Agar No. 2  12.0

48.5 grams of powder was suspended in 1 litre of distilled water, mixed well and dissolved by boiling then sterilized by autoclaving at 121˚c for 15minutes. After sterilization the medium was cooled and poured into Petri dishes.

**Positive control:** *E.coli 0157*

**Negative control:** *E.coli ATCC 25922*
Appendix (1-O):

Blood Agar Base

Formula: -

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gms/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lab-Lemco’ powder</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone Neutralised</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH 7.3 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Directions:
40 grams of medium was dissolved in 1 litre of distilled water, then dissolved by heating, then sterilized by autoclaving at 121°C for 15 minutes. After sterilization the medium was poured into sterile Petri dishes.

Appendix (1-P):

Nutrient Broth

Formula:-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gms/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lab –Lemco’ powder</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>pH 7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Directions:
13 grams of powder was dissolved in 1 litre of distilled water, mixed well and distributed in small test tubes, then sterilized by autoclaving at 121°C for 15 minutes.
Appendix (1-Q):

**Columbia blood agar base**  Himedia  M 144

**Formula:**

- Peptone, special  23.0
- Corn starch  1.0
- Sodium chloride  5.0
- Agar  15.0

44.0 grams of powder was dissolved in 1 litre of distilled water, sterilized by autoclaving at 121°C for 15 minutes. Then used for preparation of campylobacter selective medium.

Appendix (1-R):

**Aesculin bile agar**  Merck  1.11432

**Formula:**  g/litre

- Meat extract  3.0
- Peptone  5.0
- OX bile  40.0
- Aesculin  1.0
- Iron (111) citrate  0.5
- Agar-agar  14.5

64 grams of powder was suspended in 1 litre of distilled water, dissolved by heating, the medium was distributed into small test tubes and sterilized by autoclaving at 121°C for 15 minutes. After sterilization the tubes were allowed to set in slope position.

**Positive control:**  *Enterococcus faecalis*

**Negative control:**  *Streptococcus pyogenes*
Appendix (1-S):

Phenylalanine agar:

**Formula:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gms/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Dl. Phenylalanine</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

The ingredients were dissolved by heating in water bath. Then dispensed in 3 ml amounts in screw-cap-bottles. Sterilized by autoclaving at 121% for 15 minutes. Allowed to solidify in a sloped position.

Appendix (1-T):

Nutrient Agar

**Formula:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gms/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lab-Lemco’ powder</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

**pH 7.4 ± 0.2**

**Directions:**

28 grams of powder was dissolved in 1 litre of distilled water, mixed well and dissolved by heating, the medium was sterilized by autoclaving at 121°C for 15 minutes. Cooled after sterilization and poured into sterile Petri dishes.
Appendix (1-U):

Mueller Hinton Agar Conda 1058
Formula: g/litre
Beef infusion 2.0
Acid casein peptone 17.5
Corn starch 1.5
Bacteriological agar 17.0

Preparation:
38 grams of medium was suspended in 1 litre of distilled water, mixed well and dissolved by heating, the medium was sterilized by autoclaving at 121˚C for 15 minutes. Cooled to 45-50˚C then poured into sterile Petri dishes.

Positive controls:

E.coli ATCC 2592
Pseudomonas aeruginosa ATCC 27853
Staphylococcus aureus ATCC 25923

Negative control: uninoculated medium
Appendices (2)

Appendix (2-A):

Oxoid CampyGen:

Directions:

1- The inoculated plates were placed in the anaerobic jar.

2- The CampyGen foil sachet was teared and the CampyGen paper sachet was removed and immediately placed in the clip carrier within the jar.

3- The jar lid was closed immediately.

4- The jar was incubated at 42°C up to 48 hours then was examined for the presence of *Campylobacter*.

Appendix (2-B):

Oxidase Disk Himedia

DD018

Appendix (2-C):

Sodium hippurate solution:

- Sodium hippurate: 0.25 grams
- Distilled water: 25 ml

Dissolved and divided 0.4 ml in small test tubes, stored in the freezer.

Appendix (2-D):

Ninhydine reagent:

- Ninhydrine: 3-5 gm
- Acetone: 50 ml
- Butyl-alcohol: 50 ml

The ninydrine powder was dissolved in the mixture of acetone and butane, then stored in a brown bottle at room temperature.
Appendix (2-E):

0.5 McFarland Turbidity standard

Solution(1):
Barium chloride 1.175 g
Distilled water 100 m

Solution(2):
Con. sulphuric acid 1 ml
Distilled water 100 ml

Working solution:
Mix 0.5 ml of solution (1) with 99.5 ml of solution (2) (This suspension equivalent to $1 \times 10^8$ CFU/ml

Distribute in screw-capped tubes of the same size as those used in the broth culture of the organism.

Shake the standard before comparing.

Appendix (2-F):

Ferric chloride solution:

One gram of ferric chloride was dissolved in 10ml distilled water and used immediately.
Appendix (3-1)

API 20 E  preparation and reading
## Appendix (3-2)

### READING TABLE

<table>
<thead>
<tr>
<th>TESTS</th>
<th>ACTIVE INGREDIENTS</th>
<th>QTY (mg/cup)</th>
<th>REACTIONS/ENZYMES</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>9-galactosidase</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td></td>
<td>2-nitrophenyl-SD-</td>
<td>0.223</td>
<td>(Ortho NitroPhenyl-SD-</td>
<td>colorless</td>
</tr>
<tr>
<td></td>
<td>galactopyranoside</td>
<td></td>
<td>Galactopyranosidase)</td>
<td></td>
</tr>
<tr>
<td>ADH</td>
<td>L-arginine</td>
<td>1.9</td>
<td>Arginine DIHydroclase</td>
<td>yellow</td>
</tr>
<tr>
<td>LDC</td>
<td>L-lysine</td>
<td>1.9</td>
<td>Lysine DeCarboxylase</td>
<td>yellow</td>
</tr>
<tr>
<td>ODC</td>
<td>L-ornithine</td>
<td>1.9</td>
<td>Ornithine DeCarboxylase</td>
<td>yellow</td>
</tr>
<tr>
<td>CIT</td>
<td>trisodium citrate</td>
<td>0.758</td>
<td>CITrate utilization</td>
<td>pale green / yellow</td>
</tr>
<tr>
<td>H2S</td>
<td>sodium thiosulfate</td>
<td>0.075</td>
<td>H2S production</td>
<td>colorless / greyish</td>
</tr>
<tr>
<td>URE</td>
<td>urea</td>
<td>0.76</td>
<td>UREase</td>
<td>yellow</td>
</tr>
<tr>
<td>TDA</td>
<td>L-tryptophane</td>
<td>0.38</td>
<td>Tryptophane DeAminase</td>
<td>yellow</td>
</tr>
<tr>
<td>IND</td>
<td>L-tryptophane</td>
<td>0.19</td>
<td>INDole production</td>
<td>colorless</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sodium pyruvate</td>
<td>1.9</td>
<td>acetoin production (Voges Proskauer)</td>
<td>colorless / pale pink</td>
</tr>
<tr>
<td></td>
<td>Gelatin (bovine origin)</td>
<td>0.6</td>
<td>GELatinase</td>
<td>no diffusion</td>
</tr>
<tr>
<td></td>
<td>D-glucose</td>
<td>1.9</td>
<td>fermentation / oxidation (GLUcoso)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>D-mannitol</td>
<td>1.9</td>
<td>fermentation / oxidation (MANnitol)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>inositol</td>
<td>1.9</td>
<td>fermentation / oxidation (INOsitol)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>D-sorbitol</td>
<td>1.9</td>
<td>fermentation / oxidation (SORbitol)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>L-rhamnose</td>
<td>1.9</td>
<td>fermentation / oxidation (RHAmmose)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>D-sucrose</td>
<td>1.9</td>
<td>fermentation / oxidation (SACcharose)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>D-melibiose</td>
<td>1.9</td>
<td>fermentation / oxidation (MELibiose)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>amygdalin</td>
<td>0.57</td>
<td>fermentation / oxidation (AMYgdalin)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>L-arabinose</td>
<td>1.9</td>
<td>fermentation / oxidation (ARAabinose)</td>
<td>blue / blue-green</td>
</tr>
<tr>
<td></td>
<td>(see oxidase test package insert)</td>
<td></td>
<td>cytochrome-OXidase</td>
<td>(see oxidase test package insert)</td>
</tr>
</tbody>
</table>

Note: TDA test is immediate, JAMES test is immediate, and VP test is 1 + VP 2 / 10 min.
Appendix (4-A):

Identification tests of *Shigella dysenteriae*
Appendix (4-B):

Identification tests of *Shigella flexneri*
Appendix (4-C):

Identification tests of Shigella boydii
Identification tests of *Enteropathogenic E.coli 0111*

HE

Mac.

DCA

Sorbitol Mac.
Appendix (4-F):

Identification tests of *E.coli* 0157
Appendix (4-G):

Identification tests of *E.coli* 1

Mac.

DCA

HE
Appendix (4-H):

Identification tests of *E.coli* 2

Mac.

Sorbitol Mac.

XLD

HE
Appendix (4-I):

Identification tests of *Aeromonas hydrophila*
Appendix (4-I) (continue)

Identification tests of *Aeromonas hydrophila*

- β. Haemolysis on blood agar
- Susceptibility to ciprofloxacin and cephalothin
- Aesculin hydrolysis test
Appendix (4-J):

Identification tests of *Morganella morgani*

Mac.

PPA test
Appendix (4-K):

Identification tests of *Yersinia enterocolitica*
Appendix (4-L):

**Identification tests of Non – cholera vibrios**
Appendix (4-M):

Identification tests of *Campylobacter jejuni*

- Skirrow medium
- Susceptibility to nalidixic acid and cephalothin
- Hippurate hydrolysis test
Appendix(4)

Zone size interpretative chart for antimicrobial used as per CLSI

Medium : Muller Hinton agar

Inoculum : equivalent to a 0.5 Mcfarland standard

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Disk content</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxy – Clavunalic acid AMC</td>
<td>20/10 μg</td>
<td>≤ 13</td>
<td>14-17</td>
<td>≥ 18</td>
</tr>
<tr>
<td>Ceftriaxone CTR</td>
<td>30 μg</td>
<td>≤ 13</td>
<td>14-20</td>
<td>≥ 21</td>
</tr>
<tr>
<td>Cefixime CFM</td>
<td>5 μg</td>
<td>≤ 15</td>
<td>16-18</td>
<td>≥ 19</td>
</tr>
<tr>
<td>Gentamicin CN</td>
<td>10 μg</td>
<td>≤ 12</td>
<td>13-14</td>
<td>≥ 15</td>
</tr>
<tr>
<td>Ciprofloxacin CPR</td>
<td>5 μg</td>
<td>≤ 15</td>
<td>16-20</td>
<td>≥ 21</td>
</tr>
<tr>
<td>Co-trimoxazole COT 1.25/23.75 μg</td>
<td>≤ 10</td>
<td>11-15</td>
<td>≥ 16</td>
<td></td>
</tr>
<tr>
<td>Doxycycline DO</td>
<td>30 μg</td>
<td>≤ 12</td>
<td>13-15</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Nalidixic acid NA</td>
<td>30 μg</td>
<td>≤ 13</td>
<td>14-18</td>
<td>≥ 19</td>
</tr>
<tr>
<td>Erythromycin E</td>
<td>15 μg</td>
<td>≤ 13</td>
<td>14-22</td>
<td>≥ 23</td>
</tr>
</tbody>
</table>

Controls :-

*Staphylococcus aureus*  ATCC 25923

*Escherichia coli*  ATCC 25922
Appendix (6) Questionnaire

A- Interview
  1- Study number
     
  2- Date
     
  3- Clinic name ................................

  4- Age  ........................................

B- Symptoms:

  Diarrhea  .................................

  Other  .................................

Macroscopical examinations:

  Mucus  .................................

  Blood  .................................

Microscopic examinations:

  Pus cells  .................................

  RBCs  .................................