

**ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCE,
SCHOOL OF GRADUATE STUDIES, DEPARTMENT OF
MICROBIOLOGY, IMMUNOLOGY, AND PARASITOLOGY**



**PREVALENCE AND ANTIBIOTIC RESISTANCE OF
ENTERIC BACTERIAL PATHOGENS ISOLATED FROM
CHILDHOOD DIARRHEA IN AMBO TOWN PUBLIC
HEALTH INSTITUTIONS**

By: Wagi Tosisa

February, 2015

Addis Ababa, Ethiopia

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Abbreviations

APW Alkaline peptone water

ATCC American Type Culture Collection

ATPHI Ambo Town Public Health Institutions

AUML Ambo University Microbiology Laboratory

CLSI Clinical and Laboratory Standards Institute

CSA Central Statistical Agency

CUFY Children Under Five Year

DEC *Diarrheagenic E. coli*

EAEC *Enterogastric E. coli*

EBR Ethiopian Birr

EPEC *Enteropathogenic Escherichia coli*

EPHI Ethiopian Public Health Institution

OPD Outpatient Departments

Spp Species

SPSS Statistical Package for Social Science

SS Salmonella Shigella Medium

STEC *Shiga-toxin* producing *E. coli*

TCBS Thiosulphate Citrate Bile Salt Sucrose

XLD Xylose lysine desoxycholate agar

ABSTRACT

Introduction: Diarrhea particularly due to enteric bacterial pathogen is a major health problem worldwide. In developed countries, it contributes primarily to morbidity but, in the developing world like Ethiopia, it is responsible for morbidity and a high level of mortality, particularly in children below five years of age.

Objective: This study aimed to investigate enteric bacterial pathogens from children aged less than five years old with diarrhea in Ambo town public health institutions in order to determine the prevalence of the disease and antimicrobial resistance pattern.

Methodology: Stool samples from 239 children less than 5 years of age with diarrhea attending Ambo Town Public Health Institutions was examined at the Ambo University of Microbiology Laboratory, Ambo, West Ethiopia, from January to July 2014. All collected samples were processed for isolation and antibiotic susceptibility testing of *Salmonella*, *Shigella*, *Vibrio* species and other bacterial species using conventional laboratory tests. PCR was done to confirm *Salmonella* by amplifying a 496-bp genetic sequence of members of the genus *Salmonella*. Antibiogram test was performed by Kirby Bauer disc diffusion method using ten commonly used antibiotics.

Results: From the 239 children screened, enteric bacteria were isolated from 24 (10%). This included; three (1.3%) *Shigella flexneri*, two (0.8%) *Shigella boydii*, one (0.4%) *Shigella sonnei*, three (1.3%) *Salmonella* species, and fifteen (6.3%) other bacterial species. There was no *Vibrio* species isolated in this study. The highest resistance among the total enteropathogenic bacteria was observed against Ampicillin (95.8%) followed by Tetracycline (70%), Amoxicillin (62.5%), Cotrimoxazole (58.3%), Chloramphenicol (41.7%), Nalidixic acid (16.7%) and Cefotaxime (4.7%). All isolates were sensitive to Amikacin, Ciprofloxacin and Gentamycin except 3 intermediate.

Conclusion: This study suggests that *Shigella*, *Salmonella* and other enteric bacteria species were some of the pathogenic infection among children with diarrhea in ATPHI. The highest prevalence of antimicrobial resistance was to ampicillin followed by Tetracycline and Amoxicillin. Though still at low levels, the major concern from this finding is the emerging resistance of enteric pathogens that was observed to Nalidixic acid and Cefotaxime.

1. Introduction

1.1. Background

Infectious diarrhea, particularly due to enteric bacteria pathogen is a major health problem worldwide. In developed countries, infectious diarrhea contributes primarily to morbidity but, in the developing world, it is responsible for a high level of mortality, particularly in children below 5 years of age [Cheng et al., 2005]. Global, regional and national estimates clearly place diarrheal diseases as a major public health problem worldwide as it is responsible for approximately 4 billion cases of diarrhea per annum, of which 2 million cases result in death [UNICEF, 2012]. It is one of the leading causes of illness in young children in developing countries [Pavani, 2012]. Diarrheal diseases remain one of the most prevalent public health problems of today. Each year, more than 2 million children do not live to see their 5th birthday because of diarrhea and pneumonia [Boschi et al., 2009]. Diarrhea can be caused by wide range of bacteria (e.g. *Shigella* species, *Salmonella* species, *Escherichia coli* and *Vibrio cholerae*), enteroparasites (e.g. *Giardia* species and *Entamoeba histolytica*) and viruses (rotavirus, adenovirus and Norwalk virus) [Martha, 2004]. Progressive increase of antimicrobial resistance among enteric pathogens in developing countries is becoming a critical area of concern [Parashar, 2003]. Diarrhea caused by multidrug-resistant bacteria is an important public health problem among children in developing countries. Usually inappropriate prescription of antibiotics prompted resistance and increased infectious disease mortality not only in developing countries but also in developed countries [Dandekar et al., 2010].

The progressive increase in antimicrobial resistance among enteric pathogens particularly *Shigella*, *Vibrio cholerae*, *Enteropathogenic E. coli* (EPEC), *Salmonella typhi* and *S. enteritidis* species is becoming a critical concern worldwide, particularly in the developing world, where there are high rates of diarrheal diseases which are associated with mortality. It is most likely related to the frequent unrestricted use of over the counter drugs without medical supervision [Hoge et al, 1998]. The emergence and spread of antibiotic resistance in bacteria is of medical importance and positions serious constraints on the options available for the treatment of many infections. This problem has been brought into standing by the recent widespread outbreaks of enteric diseases caused by drug resistant organisms [Nys et al., 2004; Grenet, 2004]. Among

enteric pathogens, major epidemics of infection with antibiotic resistant *Shigella* have occurred in Latin America, Asia and Africa [Mata et al., 1970; Taylor et al., 1989; Gebre-Yohannes et al., 1990]. A major outbreak of multi-drug resistant *Shigella dysenteriae I*, occurring concurrently with *Vibrio cholerae* serovar O1 Ogawa was reported along the coastline of Kenya [Iijima et al., 1995]. The strain of *Sh. dysenteriae* was found to be resistant to Ampicillin, Tetracycline, Chloramphenicol, and Cotrimaxazole but sensitive to Gentamycin, Nalidixic acid and Kanamycin, apart from one strain that was resistant to Kanamycin. However most *V. cholerae* strains were sensitive to Gentamicin, Nalidixic acid, Kanamycin, Tetracycline, Chloramphenicol and a few were resistant to Ampicillin. Also multi-drug resistant *enteroaggregative E. coli* (EAEC) serotype O44 associated with acute and persistent diarrhea was reported in Kenyan children [Kariuki et al., 1994]. A number of studies done at a public teaching hospital in Kenya in 1991 and 1992 [Kruse et al., 1992; Bii et al., 2005] recorded a prevalence of over 50% to ampicillin and 80% - 100% resistance to Tetracycline among *Salmonella* and *Shigella* isolates causing nosocomial infections during the period 1986-1990.

1.2. Statement of the Problem

Diarrhea is a significant health problem worldwide, especially in the developing world where adequate sanitation facilities are lacking [Kosek et al, 2003]. Acute diarrheal disease is a major public health problem throughout the world, with over two million deaths occurring each year, and affecting mostly children under 5 years of age in developing countries. Diarrhea diseases are especially common in developing countries with poor hygiene and sanitation and with limited access to safe drinking water. Underlying conditions, such as malnutrition [Bryce J et al., 2005], which increase the risk of contracting diarrhea, are also common in these countries. These factors may result in a significant disease burden and economic effect due to direct medical costs, loss of work, lower quality of life and mortality. The etiological agents for acute diarrhea include a wide range of bacteria, virus and parasites [Mota et al., 2010; Kirkwood et al., 1991].

Infectious diarrhea affects mainly children who are at risk of complications, especially when they suffer from malnutrition, which is common in Ethiopian children. In Ethiopia according to study done in Hawassa Town, of the 158 fecal samples of childhood diarrhea, 35(22.2%) bacterial pathogens were isolated. The isolated bacteria were *Campylobacter* species, 20 (12.7%), *Shigella* species, 11 (7.0%), and *Salmonella* species, 4 (2.5%). The majority of the isolates were sensitive

to Chloramphenicol, Ciprofloxacin, Nalidixic acid and Cotrimoxazol and high rate of drug resistance was observed against Erythromycin and Amoxicillin[Getamesay et al., 2014]. Moreover, there was no available data about the spread and antibiotic resistance of the enteropathogenic bacteria for younger than 5 years of age children in Ambo. Hence, this research is in part an attempt to determine the occurrence of antimicrobial resistance for enteric pathogens isolated from childhood diarrhea among under five years age in Ambo Town Public Health Institutions, Western Ethiopia.

1.3. Significance of the study

Infectious diarrhea affects mostly children who are at risk of complications, especially when they suffer from malnutrition, which is common in Ethiopian children. Despite high prevalence of diarrheal disease among children under five years, antibiotic resistance of bacterial pathogens test is not part of routine child care in study area. Hence, this study was in part an attempt to determine the occurrence of antimicrobial resistance for enteric pathogens isolated from childhood diarrhea in Ambo, Ethiopia. The results of this study could increase clinical information in order to prevent and treat the infections. It is also serve as a steppingstone for other researches to be conducted in the future.

2.Literature Review

2.1 Childhood Diarrhea

Diarrhea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria, viruses and protozoa. However, just a few organisms are responsible for most acute cases of childhood diarrhea [WHO, 2009]. Rotavirus is the leading cause of acute diarrhea, and is responsible for about 40 percent of all hospital admissions due to diarrhea among children under five worldwide [WHO, 2008]. Other major causes are bacterial pathogens including *E. coli*, *Shigella*, *Campylobacter* and *Salmonella*, along with *V. cholerae* during epidemics [Goma, 1995]. Cryptosporidium has been the most frequently isolated protozoan pathogen among children seen at health facilities and is frequently found among HIV-positive patients [Tindyebwa, 2004]. Though cholera is often thought of as a major cause of child deaths due to diarrhea, most cases occur among adults and older children [WHO, 2006]. Socioeconomic conditions may confuse the presumed relationship between diarrhea and impaired cognition because poor or less educated families are likely to have both high rates of childhood diarrhea and delayed care seeking [Sigman et al., 1991]. The study done in Bangladesh found that, like studies conducted in other developing countries, the major pathogens of childhood diarrhea are rotavirus, *C. jejuni*, *ETEC*, *EPEC*, *Shigella* spp., and *V. cholerae*. Other similarities include the low prevalence or absence of infections due to *Salmonella* spp., *Plesiomonas shigelloides*, *G. lamblia*, *E. histolytica*, *EIEC*, and *EHEC*; a lack of association with diarrhea for *EAEC* and *DAEC*; a high prevalence of mixed infections; and a high rate of asymptomatic carriage of pathogens by controls [Brown et al., 1989; Echeverria et al., 1989; Huilan et al., 1991].

2.2 Global epidemiology of childhood diarrhea

Diarrhea remains the second leading cause of death next to Pneumonia among children under five globally [UNICEF/ WHO, 2006]. Nearly one in five child deaths – about 1.5 million each year – is due to diarrhea. It kills more young children than AIDS, malaria and measles combined [WHO, 2004]. Annual mortality from diarrhea in children less than five years old in developing countries was 1.8 million deaths and it decreased from 4.5 million deaths in the last 20 years [Black, 2007]. Africa and South Asia are home to more than 80 per cent of child deaths due to

diarrhea. Just 15 countries account for almost three quarters of all deaths from diarrhea among children under five years of age annually; India, Nigeria, Democratic Republic of the Congo, Afghanistan, Ethiopia, Pakistan, Bangladesh, China, Kenya, Niger, Burkina Faso, United Republic of Tanzania, Mali and Angola were ranked by WHO in 2009 from first to fifteen respectively [UNICEF/WHO, 2009].

2.3 Diarrheal disease in under 5 children in Ethiopia

Diarrhea has been estimated to be responsible for 25% to 75% of all childhood illnesses in Africa [Freij et al., 1979]. According to the World Health Statistics 2011, 27% of deaths of children under five years of age in Ethiopia are caused by diarrheal diseases. The study done in Nekemte town among under-five children the prevalence of diarrheal morbidity over a period of two weeks preceding was about 28.9% [Regassa G et al., 2008]. Another study done in Eastern Ethiopia showed that children who had diarrhea two weeks before the interview yielded a prevalence of 22.5%. According to that study children in the age group 6 - 11 months had the highest prevalence of diarrhea followed by the age groups 12 - 23 months [Mengistie et al., 2013]. Behavioral factors associated with acute childhood diarrhea include lack of hand-washing, poor infant and young child feeding practices and lack of child immunizations [Wondwossen, 2008]. Rotavirus is estimated to cause about 40 per cent of all hospital admissions due to diarrhea among children under five years of age worldwide [WHO, 2008]. Introduction of rotavirus vaccine in countries with the greatest diarrhea burdens, especially in Asia and Africa, must be accelerated on a priority basis. Global rotavirus vaccine introduction has recently been recommended by the World Health Organization [WHO, 2009]. The study done in Kotebe Health Center showed that occurrence of childhood diarrhea among children who started supplementary feeding before six months was around four times higher when compared with those less than six months and not started a supplementary food yet. An association has been found between hand washing after cleaning the child's bottom and diarrhea among under five children [Tilahun, et al., 2014]. Another study done in Gilgel Gibe Field Research Center, Southwest Ethiopia showed that out of the total causes of death in post-neonatal period an acute diarrheal disease accounted for 30% [Deribew et al., 2007].

2.4. Bacterial pathogens

Bloody diarrhea at childhood represents approximately 20-30% of all cases and has higher morbidity and mortality. Treatment with antibiotics is beneficial in cases of *Shigella*, *Campylobacter*, *Yersinia* and *Salmonella* infection, principally in those children with a higher risk of invasive disease [Bern et al., 1992]. Approximately 5 episodes of diarrhea per child per year occur among children under 5 years old, and ~0.2% of the cases are fatal [Shapiro et al., 2001]. Sub-Saharan Africa is among the regions with the highest morbidity and mortality from diarrheal diseases; however, detailed population-based surveillance data and antimicrobial susceptibility patterns of specific bacterial diarrheal pathogens for the area are lacking [Kirkwood, 1991].

According to the study done in Oman in the year 2002 to 2006, 11.4% showed positive result for a bacterial pathogen. *Salmonella* (5.8%) and *Shigella* species (4.4%) were the common enteric bacterial pathogens isolated. Less commonly (1.2%) include *Aeromonas hydrophila*, *E. coli*, *V. cholerae*, *Klebsiella*, *Pseudomonas* and *Plesiomonas shigelloides* [Prakash, 2008].

During a 5-year period (1995–1999) a total of 7090 stool samples obtained from patients with acute diarrhea, mostly community-acquired, were examined for bacterial pathogens, in the Greek island of Crete. One or more enteric pathogens were isolated from 987 patients (14%). *Salmonella enterica* were the most commonly isolated bacteria (6%), followed by *Campylobacter spp.* (4.2%), and *Enteropathogenic Escherichia coli (EPEC)* (1.8%). *Yersinia enterocolitica* (0.6%), *Shigella spp.* (0.3%), and *Aeromonas hydrophila* (0.04%), were less frequently isolated [Maraki et al., 2003].

In one study, which was carried out, to determine Epidemiological and Microbiological Aspects of Acute Bacterial Diarrhea in Children from Salvador, Bahia, Brazil, *Shigella spp.* was the most frequent pathogen, being found in 141 (54.3%) cultures (113, or 80.1%, were *S. sonnei* and 28, or 19.9%, were *S. flexneri*), while *Salmonella spp.* was found in 100 (38.4%) cultures and *E. coli* was found in 19 (7.3%). No typhoidal *Salmonella spp.* specimens were isolated [Santos et al., 2005].

A study done in Oman among 856 children < 12 revealed the mean age was 2.4 (SD 2.3) years; the majority (92.9%) was < 5 years. According to this study, bacterial etiology was found in 15.2% of cases; 10.6% due to *Shigella* and 2.1% to *Salmonella*. *Sh. sonnei* was the commonest

Shigella serogroup isolated. *Salmonella* infection was significantly associated with cramps, while *Shigella* infection was associated with fever, bloody stools and cramps. Antibiotics were prescribed in 36.2% of cases and the resistance to the common antibiotics tested was low [Patel et al., 2005].

Pathogenic strains of *E. coli* are a common cause of acute infectious diarrhea. *E. coli* can cause diarrhea by different mechanisms. Each type of *E. coli* diarrhea is associated with a different pathotype of *E. coli*, and each pathotype has characteristic virulence determinants that contribute to its pathogenic mechanisms. A study from Iran found most frequently identified diarrheagenic *E. coli* (*DEC*) was enteropathogenic *E. coli* (47.5%), followed by enteroaggregative (20%), enterotoxigenic (17.5%) and shiga-toxin producing *E. coli* (15%). No isolates of enteroinvasive *E. coli* were detected and among the 40 *DEC* strains 27(67.5%) were multidrug resistant [Alikhani et al., 2013].

2.5. Antimicrobial resistance

Antibiotic resistance was reported very early in the development of these wonder drugs. Sir Alexander Fleming's original report in 1929 noted that some bacteria, including the microbe now called *Escherichia coli*, were resistant to the effect of penicillin. In 1940, Edward Abraham and Ernst Chain reported the presence of an enzyme in *E. coli* that destroyed penicillin, this was several years before the drug became widely used to treat patients. In the subsequent decades, bacterial antibiotic resistance has become a widespread and well-known phenomenon [Guilfoile et al., 2007].

The study in Northern India done on stool specimens from 119 patients yielded *Shigella*, *Salmonella*, *Vibrio cholerae* or *Aeromonas*. Resistance to antimicrobial agents was common among all pathogens. Among *shigellae* an overall resistance of 63.6, 58.1 and 16.3 per cent was observed for nalidixic acid, cotrimoxazole and furazolidone respectively. Seven isolates of *Shigella* were resistant to ciprofloxacin, (18.5%) of non-typhoidal salmonellae were resistant to ciprofloxacin. *V. cholerae* were generally susceptible to tetracycline (only 1 isolate out of 13 resistant) and other drugs except nalidixic acid (89.5% resistance) and cotrimoxazole (77.8% resistance)[Taneja et al., 2004]. The resistance of enteropathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as in developed countries; resistance has emerged even to newer, more potent antimicrobial agents [Bauer et al., 1996].

Shigella spp, were isolated in 7.7% of samples from 271 children with diarrhoea in two hospitals located in southwestern Nigeria. Antimicrobial susceptibility testing showed that drug resistant *Shigella* spp. was spreading in the communities but the isolates were susceptible to new quinolones [Efuntoye et al., 2011].

Another study from Iran showed that of 1686 stool samples, 91% *Shigella* isolates were resistant to one or more antimicrobial agent (s) and 88% were multi-drug resistant. Most strains were resistant to chloramphenicol (90%), ampicillin (89%), co-trimoxazole (84%), tetracycline (83%) and nalidixic acid (51%). Resistance to amoxicillin-clavulanic acid, ceftriaxone, amikacin, nitrofurantoin and ciprofloxacin was observed in 34.9%, 23.4%, 6.6%, 3.6% and 1.8% of the isolates, respectively. Emerging resistance against nalidixic acid (42.3%) was observed [Mashouf et al., 2006].

A study was carried in four provinces of Kenya, for the period between 1 October 2007 and 30 September 2008 from a total of 651 outpatients with diarrhea who were under five years of age, the highest levels of resistance among the *E.coli* isolates were observed in ampicillin and trimethoprim/sulphamethoxazole each at 95% followed by tetracycline at 81%. *Shigella* isolate levels of resistance ranged from 80% to 100% for ampicillin, tetracycline and trimethoprim/sulphamethoxazole [Sang et al., 2012].

In Ethiopia according to study done in Jimma health center from total of 260 diarrheal sample, 129 (49.6%) were positive for intestinal parasite, *Shigella* and *Salmonella* species. *Shigella* species showed 100 % resistances to Ampicillin, Amoxicillin, and Cotrimoxazole. All *Salmonella* isolates were resistant against Amoxicillin. All *Shigella* and *Salmonella* species were susceptible to Ceftriaxone, Ciprofloxacin and Gentamycin [Beyene et al., 2014]. Another study done in Bahir Dar town, Ethiopia showed that from the total 422 stool samples, 33 (7.8%) showed positive results for *Salmonella* species. From the 33 *Salmonella* isolates, 29 (87.9%) were *Salmonella enterica* subspecies arizonae and 4 (12.1%) were *Salmonella group-A*. *Salmonella* isolates were highly resistant to Ampicillin (93.9%) followed by Augmentin (75.8%) and Trimethoprim/Sulfamethoxazole (48.5%). However, the isolates showed high susceptibility to Ciprofloxacin and Norfloxacin (93.9% each) followed by Gentamicin (87.9%). Likewise, the *Salmonella* isolates showed 90.9% of multidrug-resistance. *Salmonella enterica*

subspecies arizonae were the dominant strains of *Salmonella* isolated from children with acute diarrhea in that study [Yemane et al., 2014].

3. Objectives of the study

3.1. General objective

This study aimed to investigate enteric bacterial pathogens associated with pediatric diarrhea and their antimicrobial resistance in Ambo town public health institutions

3.2. Specific objective

1. To identify enteric bacterial pathogens (*Salmonella* species, *Shigella* species, *Vibrio* species and other enteric bacterial strain)in fecal samples from children with diarrhea
2. To assess the antibiotic susceptibility pattern of isolated bacteria.

4. Materials and Methods

4.1. Study design

Institutional based, cross-sectional study was carried out to determine common bacterial pathogen of diarrheal diseases in children under five years of age and their antibiotic susceptibility.

4.2. Study area and period

The study was conducted in Ambo town public health institutions; Ambo Hospital and two Health Centers (Ambo HC and Awaro HC) from January to July, 2014. These institutes give a health service for Ambo Town and Ambo Zuria woredas.

Ambo Zuria is one of the woreda in Western Shewa Zone, Oromia Region of Ethiopia. It is bordered on the southwest by Tikur, on the west by Cheliya, on the north by Ginde Beret, on the

northeast by Jeldu, on the east by Dendi, and on the southeast by the Southwest Shewa Zone. The administrative center of this woreda is Ambo. Ambo is located 110 KM west of Addis Ababa at a latitude and longitude of 8°59'N 37°51'E and an elevation of 2101 meters; Other towns include Gorosile and Meti. Ambo Zuria and Toke Kutaye woredas and Ambo town were part of former Ambo woreda.

The 2007 national census reported total populations for this Woreda to be 108,406, of whom 54,186 were men and 54,220 were women; 865 or 0.8% of its population were urban dwellers. The majority of the inhabitants 51.82% said they practiced Ethiopian Orthodox Christianity, while 32% of the population practiced traditional religions, and 15.9% were Protestant[CSAE, 2007].

4.3. Source Population

All children with diarrhea disease in Ambo town and Ambo Zuria Woreda

4.4. Study population

Under five years of age children with diarrheal disease who attended Ambo town Public Health Institutions Pediatric OPDs and Pediatric wards in Ambo Hospital and two Health Centers (Ambo HC and Awaro HC) during the study period.

4.4.1 Inclusion Criteria

- Children less than or equal five years of age with diarrheal disease
- Children who were not on antibiotics for current diarrheal attack

4.4.2. Exclusion Criteria

- Age above five years
- Critically ill and unable to give required sample
- Children received any antibiotic within 2 weeks before the beginning of the diarrhea

4.5. Variables

4.5.1 Dependent Variables

Bacterial isolates and antimicrobials susceptibility

4.5.2. Independent Variables

Gender, age, number of household member, number of rooms in the house, number of household workers, domestic animals in the house, drinking water source and clinical data

4.6. Sample Size

The sample size for the study was determined using a single population proportion formula. The study assumed the prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhea in Kenya is 115 (17.7%), [Sang et al., 2012] at 95% level of confidence and 0.05 margin of error. Then, the totals of 250 children diarrheal patients were targeted to be included in the study.

$$n = \frac{(Z\alpha)^2 (pq)}{d^2}$$

d^2

Where: n = sample size

$Z\alpha/2$ = level of confidence

P = diarrhea prevalence

$q = 1-p$

d^2 = margin of error (0.05)

$$n = \frac{(1.96)^2 (0.18) (1-0.18)}{(0.05)^2}$$

$$(0.05)^2$$

$$= \frac{(3.8416)(0.18)(0.82)}{0.0025}$$

$$0.0025$$

$$= 227 + 10\% \text{ non-response rate}$$

$$= 250$$

4.7. Sampling Procedure

The study subjects were selected following systematic random sampling from one public Hospital and Two HC of Ambo Town among less than five children with diarrheal disease attending pediatric OPD and admitted to hospital pediatric ward during the study period.

4.8. Sample collection, handling and transport

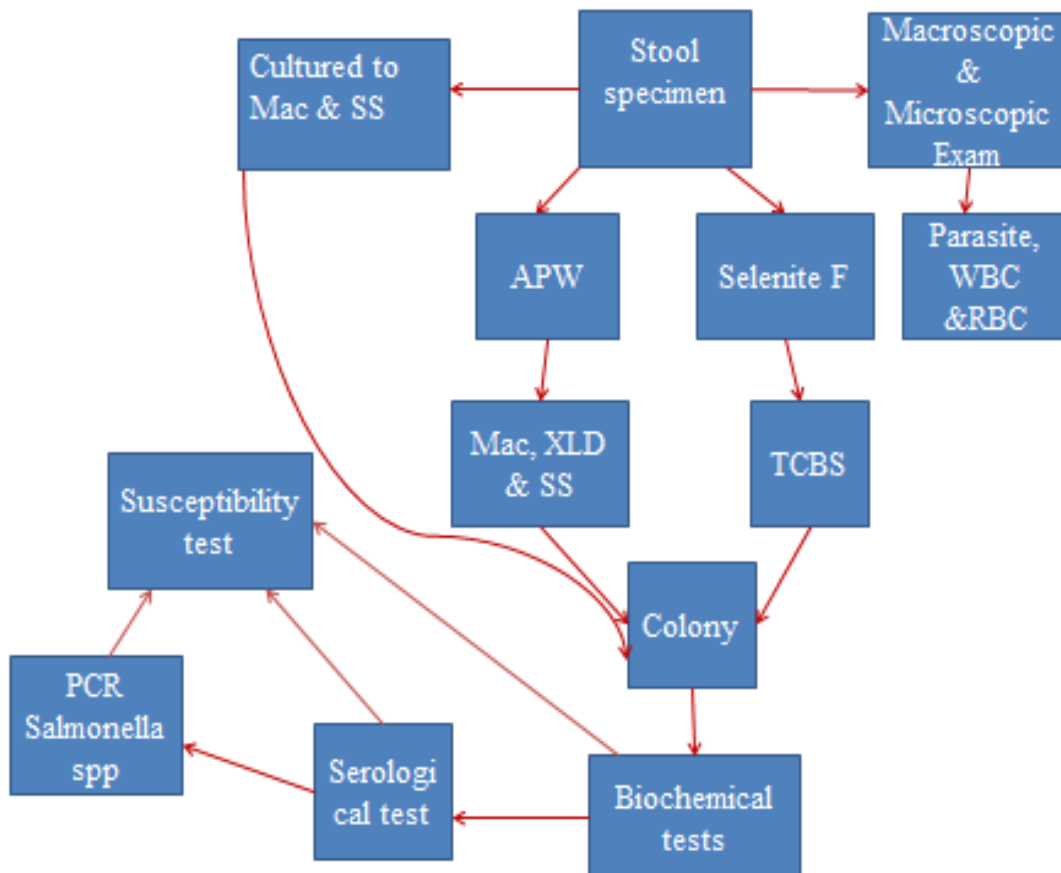
Specimens were collected during workdays at hospital and HC site. A stool specimen and information on patient demographics, medication use before the Hospital and HC visit, and report symptoms was obtained from parents/guardians of every child. Patient's demographic data was recorded which included name, age, sex and date of specimen collection. Body temperature measuring, demographic data and stool specimens were collected after physical examination.

A single diarrhea stool (defined as three or more loose stools accompanied with mucous in a 24-hrs period or any number of watery stools as per WHO definition)[UNICEF/WHO, 2009] was collected from 239 children. The majority of samples were from children diagnosed at Ambo Hospital due to high client flow. Stool samples were collected by trained healthcare personnel using sterile stool containers, free from urine and transported to the Microbiology Laboratory at the Ambo University, in ice packs and were processed within 4 hours of collection. The samples collected were well labeled and ready for analysis. Stools samples were rejected while they were unlabeled, delayed above 4 hours, transported without ice bag, contaminated with urine and dried out. All specimens arrived at the laboratory within 4 hours of collection were processed in the same day. In case of delayed sample for more than four hours; Cary- Blair's transport medium (CA, USA) was used to preserve samples at 4°C and it was processed within less than 24 hours.

4.9. Laboratory Method: All stool specimens were cultured at AUML for isolation of *Shigella* species, *Salmonella* species, *Vibrio* species and other enteric bacteria. The sample obtained was inoculated aerobically, incubated at 37°C for 18 - 24 hours in Selenite F broth (HIMEDIA, India) and alkaline peptone water then to Xylose lysine desoxycholate agar (XLD) (OXOID, England) and Thiosulphate citrate bile salt sucrose (TCBS) (SRL, India) respectively. Alkaline peptone water was used for the enrichment of *Vibrio* spp. whereas Selenite F broth was used for the enrichment of *Salmonella* spp. and *Shigella* species. It was also directly inoculated into MacConkey agar (SRL, India) and *Salmonella-Shigella* (SRL, India) agar, incubated at 37°C for 18 - 24 hours. The colonies of each representative isolates was then characterized using standard

bacteriological method. Thiosulphate-citrate-bile salt sucrose (TCBS) agar used for the isolation of vibrio species, MacConkey agar for most enteric bacteria to characterize their lactose utilization property, XLD (*Shigella*: red colonies, *Salmonella* red with/without a black center), and SS agar for the isolation of *Shigella* and *Salmonella* species.

Figure 1 Over all Laboratory process



Biochemical screening media were used for identification of bacterial isolates. The biochemical media used were Nutrient broth (CONDA, Spain), lysine iron agar (LIA) (OXIOD, England), MRVP, Simmons citrate agar (HIMEDIA, India), Kligler iron agar (KIA) (SRL, India), Sulfide-Indole-Motility (SIM), Identification sticks Oxidase (OXIOD, England), Urea broth base (OXIOD, England), Motility Indole Ornithine Medium (MIO) (OXIOD, England), 3% H₂O₂ and Kovac's reagent were used to identify *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp and other enteric bacteria. Tests performed to isolate the enteric gram negative bacteria include gram stain morphology, pigment production, motility, urease, citrate, hydrogen sulfide utilization, oxidase, catalase, indole, Lysine, and sugar fermentation. Identification was confirmed by using polyvalent

Test procedure of slide agglutination for *Salmonella* and *Shigella* species by antisera was done by following the manufacturer instruction (see Annex V).

Polymerase chain reaction (PCR) *Salmonella* genus was checked by PCR method using specific oligonucleotide primer for it. Extractions of DNA was obtained from isolated bacteria colony and done at Aklilu lemma Institute of Pathobiology.

Bacteria were grown in Selenite F broth medium and then transferred to selective culture media (XLD and SS). Colonies of salmonella characterized were checked by conventional biochemical tests. Bacterial colony suspensions were centrifuged at 500 x g for 30 min to pellet cells. Cells were re-suspended in 150 µl of lysing solution containing 50 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris HCl, 20% sucrose, and 100 µg of lysozyme, and the suspension was incubated at 37 °C for 30 min. Concentrations of DNA were determined with a fluorometer and samples were diluted with water to a concentration of 50 ng/µl for the PCR.

PCR reaction Oligonucleotide primers of 25 bp defined the amplified region of a 496-bp segment of the histidine transport operon of *Salmonella typhimurium*. This gene was selected because it was considered to be highly conserved among species of *Salmonella* [Cohen et al., 1993]. The PCR primers were designed using a software program [Rychlik et al., 1989] and synthesized in the Department of Biology at Texas A and M University. The prime sequences for the upper and lower oligonucleotides, from 5' to 3', were as follows: upper strand, ACTGGCGT-TATCCCTTTCTCTGGTG; lower strand ATGTTGT-CTGCCCTGGTAAGAGAA PCR mixture was prepared consisting of 1.0 µM of upper and lower primers, 0.2 mM dNTPs, 10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin (w/v), 0.1% Triton X-100, and 1.25 units of Taq polymerase. Forty-eight microliters of the cocktail was added to 100 ng of bacterial DNA per reaction. The reaction was run for 30 sec at 94 °C, 30 sec at 60 °C, and 45 sec at 72 °C for 30 cycles in a thermocycler. Products of the PCR were electrophoresed on a 2% agarose gel containing ethidium bromide and photographed. Positive results were indicated by the presence of a 496-bp band seen on the gel with an ultraviolet transilluminator.

4.10. Antimicrobial susceptibility: testing was done at the AUML on all bacterial pathogens by use of the disk diffusion method. An antibacterial susceptibility test was performed on Mueller-Hinton agar (SRL, India) using Kirby-Bauer technique. The zone of inhibition was measured by a ruler, recorded and compared with Clinical and Laboratory Standards Institute (CLSI) chart (Clinical and Laboratory Standards Institute) [Wayne, 2006]. The following antibiotics were used to screen for the resistance of the isolates; Amikacin (AK 30µg), Ampicillin (AM, 10µg), Amoxicillin (AX, 10µg), cotrimoxazole (SXT, 25µg), Cefotaxime (CF, 30µg), Chloramphenicol (CH, 30µg), Ciprofloxacin (CP, 5µg), Gentamycin (GM, 10µg), Nalidixic acid (NA, 30µg) and tetracycline (TTC, 30µg). All antibiotics were obtained from Oxoid Limited, Basingstoke Hampshire, UK. The zone of inhibition was then measured and the results were recorded as sensitive (s) or resistance (R) based on CLSI 9th edt CLSI. The study assumed the defining criterion for multidrug resistance (MDR) if the isolates were resistance to > 2 of the antimicrobial agents belonging to different structural classes [Bartoloni et al., 2006; Wright et al., 2000]. The plates were incubated at 37^oc for 24 hours and the zone of inhibition diameters were measured with ruler and interpreted according to CLSI [Wayne, 2006]. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the control strains.

4.11. Microscopic examinations

The stool samples were examined with the naked eye for consistency, color and atypical components such as mucous, blood and parasites. Direct microscopic examination was done by saline and iodine preparation for WBC, RBC, intestinal parasite and protozoan detection.

4.12. Data analysis procedure

After completion of data cleaning, statistical analysis was carried out. The data was entered in to EpiData 3.02 and then transferred to SPSS version 17.0 statistical software for data processing and analysis. Descriptive statistics like proportion/ frequency mean and SD for independent variables were calculated. Bivariate analysis using crude odds ratio and Pearson's Chi-square tests was used to assess the strength associations and statistical significance between independent and dependent variables. All analyses were carried at the 0.05 significance level. The data was further processed and formulated into figures and tables using Microsoft Excel version 2007 computer program.

4.13. Data quality management

The questionnaire was translated to local language and translated back into English to check its consistency. Training was given to facilitators and supervisors by principal investigator for two days on the procedures of data collection and handling of collected data. Pre-test was conducted in Guder HC before actual data collection. The drug susceptibility patterns for the quality control strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 was done as the zone of inhibition for these organisms is known in accordance with CLSI (2006). Supervision was done by the investigator and supervisor throughout the study period. The pretested responses were excluded from the study results. A data was entered into EpiData 3.02 software to minimize data entry errors.

4.14. Ethical Considerations

Ethical clearance was obtained from the Ethics Review Committee of Addis Ababa University Health Science College Department of Microbiology, Immunology and Parasitology. Formal letters were obtained from the district health office and written for each Health Facilities in order to get permission. An informed consent was obtained from the parents/guardians of every child before taking the stool samples. Positive cases were reported to attending physician or health professional. No fee was charged.

4.15. Dissemination of results

Culture results and antimicrobial susceptibility results were communicated to the Hospital and HCs officers at each health facility within 1 week of specimen collection and were used by health care providers to evaluate treatment decisions. The findings of the study will be disseminated and submitted to Addis Ababa University School of Medicine as partial fulfillment of master's degree in Medical Microbiology. The result of the study will be presented on scientific workshops and publication to scientific journal to be used by researchers. It will be disseminated to Haramaya University, Ambo University and West Showa Health Bureau.

Results

Socio-demographic characteristic of the study participants

A total of 250 children less than five years were planned to participate in the study, out of which 239 were enrolled making a response rate of 95.6%. There were 4 children families not volunteer to participate in the study and 7 stool samples were excluded due to rejection criteria. A total 239 children were included in the study, 125 (52.3%) were boys and 114 (47.7%) were girls; with male to female ratio of 1.1:1. The age ranges from 5 – 60 months with mean of 27.96 (SD \pm 17.09) months. Of the 239 children included in the study, one hundred and eighty (75.7%) were from Ambo Hospital, 34 (14.2%) were from Ambo Health Center and 24 (10%) were from Awaro Health Center. One hundred sixty two (67.8%) were from urban, while 77 (32.2%) were from rural (Table 1).

Table 1 Socio-demographic data, residence, domestic animals in house and type of diarrhea of the study group in ATPHI, Ethiopia from January - July 2014

		Frequency	%
Health Intuition	Awaro Health center	24	10.0
	Ambo Health center	34	14.2
	Ambo Hospital	181	75.7
Residence	Urban	162	67.8
	Rural	77	32.2
Gender	Male	125	52.3
	Female	114	47.7
Household workers	≥1	211	88.3
	None	28	11.7
Domestic animals in the house	Yes	97	40.6
	No	142	59.4
	Total	239	100.0
Source of drinking water	tap water	170	71.1
	Domestic well water	13	5.4
	River water	42	17.6
	Public hand pump water	14	5.9
Type of diarrhea	watery diarrhea	49	20.5
	bloody diarrhea	32	13.4
	Mucoid diarrhea	100	41.8
	Loose stool	58	24.3
Household member/room	<1.5 persons-per-room	198	82.8
	>1.5 persons-per-room	41	17.2
Antibiotics prescribed in last 4 weeks	Cotrimoxazole	10	4.2
	Ceftriaxone	2	0.8

	Amoxicillin	1	0.4
	Metronidazole	2	0.8

It was found that 198(82.8%) cases were living in households of non-overcrowded(<1.5persons-per-room) whereas41(17.2 %)living with overcrowded household members (>1.5 persons-per-room)[Maryland et al., 2007]. There were ≥ 1 worker(s) among the family members in 211 (88.3%) of the respondents household whereas 28 (11.7%) haven't any work. Among the respondents, 177(74.1%) had one child under five year, 59(24.7%) had two children and only 3(1.3%) had three children. Among the children with diarrhea 97(40.6%) households had domestic animals in their house; 82(34.3%) had poultry, 43(18%) goat, 38(15.9%) sheep, 73(30.5%) cattle and 49(20.5%) donkey (Table 1).

Regarding drinking water source, 170 (71%) used tap water, 42(17.6%) river water, 14(5.9%) used public hand pump water and 13(5.4%) used domestic well. Among the total respondents, in the last 4 weeks antibiotics was given for15(6.3%) of children with diarrhea. Only four antibiotics were prescribed in the treatment for the past history of illness: Cotrimoxazoale, Ceftriaxone, Amoxicillin, and Metronidazole (Table 1); cotrimoxazoale was the most commonly prescribed antibiotic.

Table 2 Age distribution of diarrhea & bacterial isolate in children ≤ 5 years at in ATPHI, Ethiopia from January -July 2014

Age in months	Stool Culture		(%)	Total
	Positive	Negative		
0-6	1	10	4.6	11
7-12	4	41	18.8	45
13-24	9	62	29.7	71
25-36	2	31	13.8	33
37-48	3	31	14.2	34
49-60	5	40	18.8	45
Total	24	21	100.0	2 9

Clinical characteristics

The clinical complaints recorded include fever by 78.2%, chills by 21.3%, vomiting by 66.1%, bloody diarrhea by 13.4%, mucoid diarrhea by 41.8%, watery diarrhea by 20.5%, loose stool by 24.3% and weight loss by 34.7% among the total respondents as shown in Figure 2.

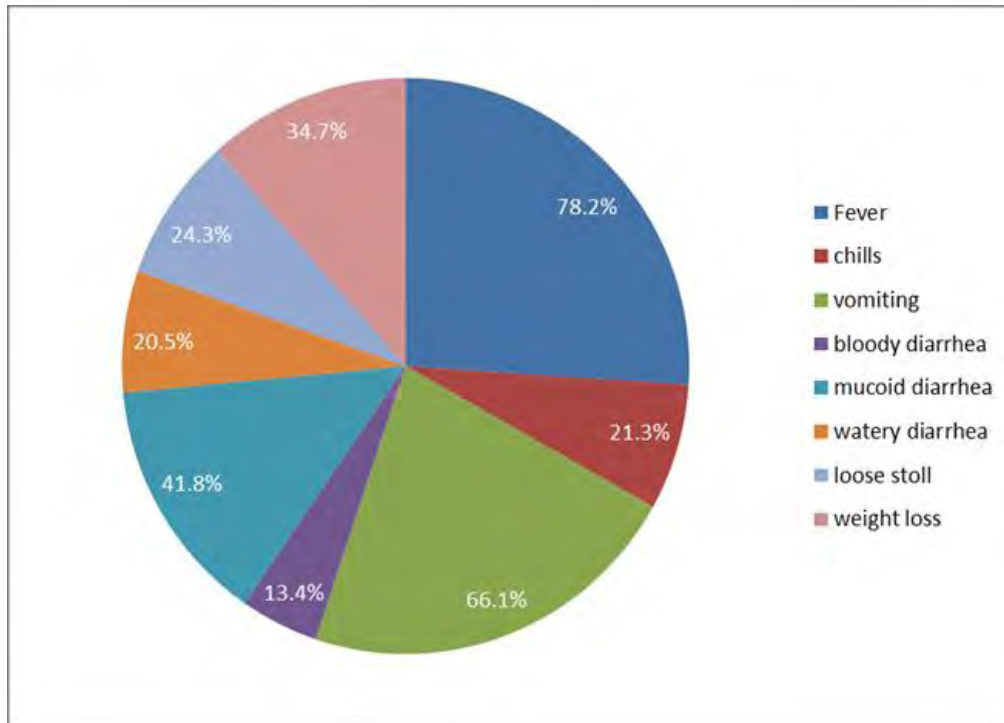


Figure 3 clinical characteristics of children with diarrhea at ATPHI in January - July 2014

Isolation of enteric bacterial pathogens

From the total 239 stool specimens, isolated enteric bacteria were *Shigella flexneri* 3 (1.3%), *Shigella boydii* 2 (0.8%), *Shigella sonnei* 1 (0.4%), *Salmonella* species 3 (1.3%), and other bacterial pathogens 15 (6.3%). Those other bacterial species are positive for culture media and conventional biochemical characteristics of *E. coli* but due to lack of reagent never checked by serological or PCR method. There is no *Vibrio* species isolated in this study from a total of 239 stool sample processed. From the positive stool sample 21 (87.5%) had single infection of bacterial pathogen whereas 3 (12.5%) had multiple infection of bacterial pathogen in which each

had 2 different bacterial isolates[other bacterial strains with *Salmonella*, other bacterial strains with *Shigella* and *Shigella* with *Salmonella*] (Table 3).

Table 3 Distribution of the pathogens in diarrheal stools according to patients' age in ATPHI, Ethiopia from January - July 2014

<i>Salmonella</i> spp		<i>Shigellia</i> spp			Other	<i>Vibrio</i> spp	Total
		SF	SB	SS			
0-6	-	-	-	-	1(0.4%)	-	1(0.4%)
7-12	1(0.4%)	-	-	-	3(1.3%)	-	4(1.7%)
13-24	1(0.4%)	2(0.8%)	1(0.4%)	-	5(2.1%)	-	9(3.8%)
25-36	1(0.4%)	-	-	1(0.4%)	-	-	2(0.8%)
37-48	-	1(0.4%)	-	-	2(0.8%)	-	3(1.3%)
49-60	-	-	1(0.4%)	-	4(1.7%)	-	5(2.1%)
Total	3(1.3%)	3(1.3%)	2(0.8%)	1(0.4%)	15(6.3%)		24(10%)
		6 (2.5%)					

Key: *Shigella flexneri* (SF), *Shigella boydii*(SB), *Shigella sonnei* (SS), and none (-).

Shigella and *Salmonella* species were examined by culture and confirmed by biochemical and antisera. Additionally, *Salmonella* spp. was confirmed by PCR method at Aklilu lemma Institute of Pathobiology. The PCR test was done for four samples that were suspected as *Salmonella* based on their colony characters and biochemical property and we confirmed positive for three salmonella genus. The predicted amplification product of 496 bp was seen as a band on 2% agarose gels. The band was specific for genus of salmonella and clear lane indicates negative identification of *Salmonella* species (Figure 4).

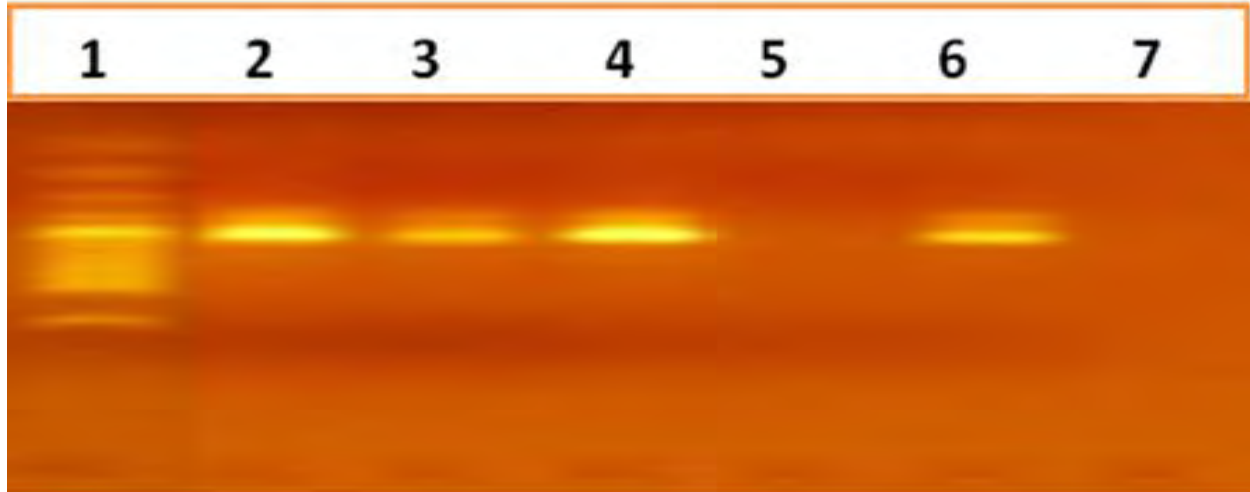


Figure 4: Ethidium bromide-stained 2% agarose gel showing results of electrophoresis of products of the PCR reaction. A 496-bp band is seen in each lane with the product of the PCR for *Salmonella* species; bands are not seen in negative lanes. A 496-bp band is seen in lane 2, 3 and 4 with the product of the PCR for genus *Salmonella*. Description: Lane 1=ladder, Lane 2-5 Clinical Isolate Lane 6= Positive control Lane 7= Negative control.

Among the children with diarrhea, *Giardia lamblia* was identified in eight (3.3%), *Entamoeba histolytica/disparin* five (2.1%), *Ascaris lumbricoides* in four 4 (1.7%), *Hookworm ova* in one (0.4%) and *Hymenolepis spp* parasites in one (0.4%) of the cases as shown in Table 4. Fecal *leucocytes* were seen in 113(55.6%) of the cases and isolates whereas RBC observed in 55(23%) of the diarrheic children.

Table 4 Intestinal parasites in children with diarrheal at ATPHI, Ambo, Ethiopia, from January - July 2014

Age in Month	Intestinal parasites (n, %)					Total
	<i>Giardia lamblia</i>	<i>Entamoeba histolytica</i>	<i>Ascaris lumbricoides</i>	<i>Hookworm ova</i>	<i>Hymenolepis spp</i>	
0-6	-	-	-	-	-	-
7-12	-	-	-	-	-	-
13-24	-	-	-	-	-	-
25-36	1(0.4)	-	1(0.4)	-	-	-
37-48	4(1.7)	-	1(0.4)	-	-	-
49-60	3(1.3)	5(2.1)	2(0.8)	1(0.4)	1(0.4)	-
Total	8(3.3)	5(2.1)	4(1.7)	1(0.4)	1(0.4)	19

Correlations of types of diarrhea and enteric bacteria isolates

Enteropathogenic bacteria were isolated with higher frequency from children with mucoid diarrhea (6.7%) and the least with loose stool (0.4%). A statistical significant association was not found between type of diarrhea and enteric bacteria isolate (Table 5).

Table 5 Types of diarrhea in patients among positive for *Salmonella*, *Shigella* and other infectionin ATPHI, Ethiopia from January - July 2014

Types of diarrhea	Types of isolate (n, %)					Total (n, %)
	<i>Salmonella</i> spp	<i>Shigella</i> spp			Other bac. spp	
		SF	SB	SS		
Watery diarrhea	1(0.4)	1(0.4)	-	-	2(0.8)	4(1.7)
Bloody diarrhea	-	1(0.4)	1(0.4)	-	1(0.4)	3(1.3)
Mucoid diarrhea	2(0.8)	1(0.4)	1(0.4)	1(0.4)	11(4.6)	16(6.7)
Loose stool	-	-	-	-	1(0.4)	1(0.4)
p. value	0.588	0.246			0.262	

Key:*Shigella flexneri* (SF), *Shigella boydii*(SB, *Shigella sonnei* (SS) and (-) none.

Antimicrobial susceptibility of isolated enteropathogenic bacteria

The antimicrobial profile for isolated enteropathogenic bacteria to various antimicrobial drugs was determined by the disk diffusion method following the recommendations of the CLSI [Wayne, 2006]. Resistance of antimicrobial agents was common among all the bacterial pathogens isolated. Among the 24 isolates 35% were below standard resistance break points for Ampicillin, Amoxicillin, Cotrimoxazole, Chloramphenicol, Nalidixic acid, Tetracycline and Cefotaxime. The highest resistance among the total enteropathogenic bacteria was observed against Ampicillin (95.8%) followed by Tetracycline (70%), Amoxicillin (62.5%), Cotrimoxazole (58.3%), Chloramphenicol (41.7%), Nalidixic acid (16.7%) and Cefotaxime (4.7%). All isolates were sensitive to Amikacin, Ciprofloxacin and Gentamycin except the 3 intermediate.

Antimicrobial profile of all 24 isolated enteric bacteria showed high resistance rates for other bacterial strains (37.3%) followed by *Salmonella* spp. (33.3%) and *Shigella* species (30%) against the tested 10 antimicrobials. Resistance to Ampicillin was 100% for both *Salmonella* species and other bacterial strains whereas 83.3% for *Shigella* species. Resistance to Tetracycline was 73.3% for other bacterial strains and 66.7% for both *Salmonella* and *Shigella* species. Resistance to Amoxicillin was observed in 80% of the other bacterial strains, 50% of the *Shigella* species but non for *Salmonella* species. Resistance to co-trimoxazole was 66.7% for

Salmonella, 60% other bacterial strains and 50% for *Shigellia* isolates. Resistance to chloramphenicol was 66.7% for salmonella, 40% for other bacterial strains isolates and 33.3% *Shigella* species. Resistance to Nalidixic acid was 20% for other bacterial strains, 16.7% for *Shigellia* species and non for *Salmonella* spp. Resistance to cefotaxime was 33.3% for *Salmonella* while non for *Shigellia* and other bacterial strains isolates (Table 6).

Among culture positive isolates there were 2(8.3%) resistance for 1 antibiotic, 3(12.5%) for 2 antimicrobial, 5(20.8%) for 3 antibiotic, 9(37.5%) for 4 antibiotic and 5(20.8%) for 5 antibiotic agents tested. Therefore, according to this study, 5 *Shigella* spp, 2 *Salmonella* spp and 15 other bacterial strains showed multi antimicrobial agent resistance (Table 6).

Table 6 Antimicrobial susceptibility of isolated enteropathogenic bacteria (*Salmonella*, *Shigellia* and other) bacteria in ATPHI, Ethiopia from January -July 2014

Enteropathogenic Bacteria (n=24)	Antibiotics types in percentage (%)										%	Total No. (%)
	AK-30	A-10	AML-2	SXT-25	CTX-5	C-30	CIP-5	GM-10	N-A	Te-30		
<i>Salmonella</i> spp. Samp. coded A	S	R	I	R	S	R	S	S	S	R	40	33.3
<i>Salmonella</i> spp. Samp. coded B	S	R	S	R	R	R	S	S	S	R	50	
<i>Salmonella</i> spp. Samp. Coded C	S	R	S	S	S	S	S	S	S	S	10	
<i>Shigellia flexneri</i>	S	R	R	I	S	S	I	S	S	R	30	30
<i>Shigellia flexneri</i>	S	R	I	I	S	I	S	S	S	S	10	
<i>Shigellia flexneri</i>	S	R	I	R	S	S	S	S	S	S	20	
<i>Shigellia sonnei</i>	S	I	R	R	S	S	S	S	S	R	30	
<i>Shigellia boydii</i>	S	R	I	R	S	R	S	S	R	R	50	
<i>Shigellia boydii</i>	S	R	R	S	S	R	S	S	S	R	40	
Other	S	R	R	I	S	R	S	S	S	S	30	
Other	S	R	R	R	S	R	I	S	S	I	40	37.3
Other	S	R	R	I	S	S	S	S	S	R	30	
Other	S	R	R	I	S	S	S	S	S	I	20	
Other	S	R	R	I	I	I	S	I	S	R	30	
Other	S	R	S	R	S	R	S	S	S	R	40	
Other	S	R	R	R	S	S	S	S	S	R	40	
Other	S	R	R	R	S	R	S	S	I	I	40	
Other	S	R	R	R	S	S	S	S	I	R	40	
Other	S	R	R	R	S	S	S	S	S	R	40	
Other	S	R	I	I	S	I	S	S	S	R	20	

Other	S	R	R	R	S	S	S	S	R	R	50	
Other	S	R	R	R	S	R	S	S	S	R	50	
Other	S	R	R	S	S	R	S	S	R	R	50	
Other	S	R	S	R	S	S	S	S	R	R	40	
Total resistance	0	95.8	62.5	58.3	4.17%	41.7	0	0	16.7	70.8	35	

Key: Amikacin (AK-30µg), Ampicillin (A-10 µg), Amoxicillin (AML-10 µg), Cotrimoxazole (SXT -25 µg), Cefotaxime (CTX-30 µg), Chloramphenicol (C-30 µg), Ciprofloxacin (CIP-5 µg), Gentamycin (GM-10µg), Nalidixic acid (NA-30 µg), Tetracycline (Te-30 µg), sensitive (S), resistance (R) and intermediate (I).

Table 7 percentage of resistance to antibiotics

(n, %) resistance to antibiotics											
Pathogen (n)	A K-30	A-10	AML-2	CM-25	CTX-5	C-30	CI P-5	G M-10	NA	Te-30	
<i>Salmonella</i> spp.(3)	-	3(12.5)	1*	2(8.3)	1(4.2)	2(8.3)	-	-	-	2(8.3)	
<i>Shigella</i> spp (6)	SF(3)	3(12.5)	1(4.2), 2*	-, 2*	-	1*	-	-	-	1(4.2)	
	SB(2)	1(4.2)1*	1(4.2), 1*	1(4.2)	-	2(8.3)	-	-	1(4.2)1*	2(8.3)	
	SS(1)	1*	1(4.2)	1(4.2)	-	-	-	-	-	1(4.2)	
Other bacterial strains	-	15(62.5)	12(50), 1*	10(41.7), 5*	-,1*	6(25),2*	-,1*	-,2*	3(12.5),1*	11(45.8)1*	
Total	-	23(95.8)1*	15(62.5)5*	14(58.3)7*	1(4.2)1*	10(41.7)3*	1*	-3*	4(16.7)2*	17(70.8)1*	

Key: Amikacin (AK-30µg), Ampicillin (A-10 µg), Amoxicillin (AML-10 µg), Cotrimoxazole (CM-25 µg), Cefotaxime (CTX-30 µg), Chloramphenicol (C-30 µg), Ciprofloxacin (CIP-5 µg), Gentamycin (GM-10µg), Nalidixic acid (NA-30 µg), Tetracycline (Te-30 µg), *Shigella flexneri* (SF), *Shigella boydii*(SB), *Shigella sonnei* (SS), none(-) and Intermediate(*).

Discussion

This is a cross-sectional study report on the prevalence and antibiotic pattern of two bacteria (*Salmonella*, *Shigella*) and other bacterial species isolated from children \leq five years with diarrhea at Ambo Town Public Health Institutions. Previous studies in Ethiopia have addressed on the prevalence and antibiotic resistance of enteric bacterial pathogens [Getamesay et al., 2014; Beyene et al., 2014; Yemane et al., 2014; Mache, 2001]. In this study the overall prevalence of enteric bacteria isolated was 10% comparable with study done in Jimma southwest Ethiopia which count 8.4% [Beyene et al., 2014], Rural Coastal India which count 11.2% [Ballal, 2014], in children from Salvador, Bahia, Brazil which count 13.1% [Santos et al., 2005], but lower than previous findings from Hawassa Town, South Ethiopia which was count 22.5% and other studies in Nigeria and Kenya [Getamesay et al., 2014; Sang et al., 2012; Cajetan et al., 2009; Karambu et al., 2013]. This might be partly due to diarrhea caused by other potential enteric pathogens such as *Rota virus*, *Campylobacter* spp, *Yersinia enterocolitica*, *Aeromonas* spp, *helminthes* and *protozoans*.

According to this study results, children between 13-24 month old are more susceptible to infectious diarrhea and enteropathogenic bacteria as demonstrated by a higher frequency enteric bacteria isolated from patients belonging to this age group (Table 2). Many studies agree with this finding that children ages between 13-24 months old are more susceptible to infectious diarrhea [Getamesay et al., 2014; Cajetan et al., 2009; Karambu et al., 2013; Heidary et al., 2014]. This might be due to children at this age try to masticate everything with their mouths. The isolation of *Shigella* spp 2.5% in this study was comparable with study done in Jimma, southwest Ethiopia which count 2.3% [Beyene et al., 2014], and Addis Ababa which count 3.2% at but lower than Jimma which count 8.8% from the same study [Beyene et al., 2011]. It also lower than other study done in Jimma, which count 20%, study done in Butajira central Ethiopia which count (4.5%), study done in Rural Coastal India which count 4.2%, and study done at Children from Salvador, Bahia, Brazil which count 54.3% [Mache, 2001; Mengistu et al., 2014; Ballal, 2014; Santos et al., 2005] respectively. Among shigella isolates, *Shigella flexneri* (12.5%) was the most dominant followed by *Shigella boydii* (8.3%), and *Shigella sonnei* (1.4%) which comparable with study done in north India [Taneja et al., 2004] but not agree with the study done from children at Jimma, southwest, Ethiopia and from Salvador, Bahia, Brazil [Mache, 2001; Santos et al., 2005]. The discrepancy might be difference in laboratory techniques.

Salmonella species (1.3%) identified from this study is comparable with study done in north India which count (1.6%) [Taneja et al., 2004], but lower than a study done in Bahir Dar Town, Ethiopia which count 7.8% [Yemane et al., 2014], a study done in Jimma southwest Ethiopia which count 6.2% [Beyene et al., 2014], a study done on multidrug resistant *Salmonella* children in Jimma which count 2.5% and in Addis Ababa which count 6.7% [Beyene et al., 2011], in Hawassa 2.5% [Getamesay et al., 2014], in Butajira which count 10.5% [Mengistu et al., 2014]. The reason might be difference in methodology used. The widespread use of antimicrobial agents in the treatment of infections in the tropics has led to serious problems of antimicrobial resistance. The emergence and spread of antimicrobial resistance in bacteria of medical importance imposes serious constraints on the options available for treatment of many infections, and this raises a concern among general practitioners and pediatricians in developing countries [Santos et al., 2005]. The resistance of enteric pathogens to currently used antimicrobial agents has increased all over the world as a result of widespread use of antimicrobials. There are several reports on multiple antimicrobial resistances among strains of pathogenic *Shigellia* and *Salmonella* species in Ethiopia [Getamesay et al., 2014; Beyene et al., 2014; Yemane et al., 2014; Mache, 2001; Mengistu et al., 2014; Roma et al., 2000; Beyene et al., 2011].

All *Shigellia*, *Salmonella* and other bacterial strains species isolates from this study displayed resistance to one or more antimicrobials including Ampicillin, Tetracycline, Amoxicillin, Cotrimoxazole, Nalidixic acid and Cefotaxime. Among the isolates there were no resistances for Amikacin, Ciprofloxacin and Gentamycin except 1 and 2 intermediate for Ciprofloxacin and Gentamycin respectively which comparable with those studies [Santos et al., 2005; Getamesay et al., 2014; Karambu et al., 2013; Ballal, 2014; Reda et al., 2011]. The highest prevalence antibiotic resistance of *Shigellia* spp against Ampicillin (83.5%) observed was comparable with the study done on *Shigellia* isolates in Awassa which count 93% [Roma et al., 2000], in Jimma which count 70.1% [Mache, 2001], in Gondar which count 79.9% [Yismaw et al., 2006], in Harar which count 100% [Reda et al., 2011], in Jimma which count 100% [Beyene et al., 2014] and Southwestern Nigeria which count 90.5% [Efuntoye et al., 2011]. This may be show widespread of resistance strain throughout the countries. It is higher than a study done in Hawassa which count 63.6% [Getamesay et al., 2014] and in Butajira which count 47.1% [Mengistu et al., 2014]. This may be due to different susceptibility method used.

Antibiotic resistance of *Shigellia* spp against Tetracycline (66.7%) was comparable with a study done in Jimma which count 63.6% [Mache, 2001], in Harar which count 70.6% [Reda et al., 2011] but lower than a study done in other parts of the Ethiopia: in Butajira (82.4%) [Mengistu et al., 2014], in Gondar University teaching hospital 86% [Tiruneh et al., 2009], in Awassa which count 90% [Roma et al., 2000], and in Gonder which count 86% [Yismaw et al., 2006]. This may be due to those strains moderately susceptible for Tetracycline at certain corner of the country. Cotrimoxazole showed 50% resistance against *Shigellia* which is comparable with a study done at Awassa (56.0%) [Roma et al., 2000], and in Addis Ababa (45.7%), [Asrat, 2008]. Fifty percent of *Shigella* species showed resistance against Amoxicillin was not comparable with study done in Hawassa [Getamesay et al., 2014], in Harar [Reda et al., 2011], in Jimma [Beyene et al., 2014] which count 100% and Southwestern Nigeria which count 81% [Efuntoye et al., 2011]. This may be due to the different laboratory techniques used for susceptibility test.

Antibiotic resistance against chloramphenicol 33.3% in this study is comparable with study done in Harar which count 29.5% [Reda et al., 2011] and in Butajira which count 29.4% [Mengistu et al., 2014], but lower than a study done in Gondar University Hospital, northwest Ethiopia [Yismaw et al., 2006], in Awassa which count 63.3% [Roma et al., 2000] and in Jimma 40.3% [Mache, 2001]. This difference is attributed to used susceptibility test. This result is also not comparable with study done in Southwestern Nigeria which counts 85.5%. This may be due to geographical difference.

Antibiotic resistance against Nalidixic acid 16.7% by *Shigellia* species in this study is comparable with study done in Jimma which count equal amount 16.7% [Beyene et al., 2014]. But higher than study done in Awassa which count 10% [Roma et al., 2000], in Jimma which count 6.5% [Mache, 2001], in Butajira which count 5.9% [Mengistu et al., 2014] and Southwestern Nigeria which count 9.5% [Efuntoye et al., 2011]. Among the *Shigella* species, none was resistant to Amikacin, Ciprofloxacin and Gentamycin comparable with study done in Harar [Reda et al., 2011], Jimma [Beyene et al., 2014] and Rural Western Kenya [Shapiro et al., 2001].

In this study resistance of *Salmonella* species to ampicillin, cotrimoxazole, Chloramphenicol, Tetracycline and Cefotaxime was 100%, 66.7%, 66.7%, 66.7% and 33.3% respectively. According to this study finding, high level of *Salmonella* resistance was observed to

ampicillin (100%) which is comparable with a study done in Harar which count 100% [Reda et al., 2011], in Bahir Dar which account 93.9% [Yemane et al., 2014] and Addis Abeba - Jimma which count 82.3% [65], but higher than study done at Hawassa which count 0% [Getamesay et al., 2014], in Jimma 62.5% [Beyene et al., 2014] and in Butajira 60% [Mengistu et al., 2014]. This high amount of resistance in different studies at different part of this country may be due to misuse of this antibiotic.

There was no resistant salmonella isolate against Amoxicillin which is comparable with study done in Hawassa [Getamesay et al., 2014]. But extremely difference with study had done in Harar which count 100% [Reda et al., 2011], in Addis Ababa – Jimma 82.3% [Beyene et al., 2011] and in Jimma count 62.5% [Beyene et al., 2014]. This difference may be due to difference in laboratory methods used. Even if a reduced level of resistance was detected for chloramphenicol, cotrimoxazole and tetracycline (66.7%) while compared to ampicillin, a relatively similar pattern of resistance (39.9% - 81.4%) was reported from study in Harar [Reda et al., 2011] and Addis Ababa, Jimma [Beyene et al., 2011]. However, lower level (0% - 48.5%) were reported from studies in Jimma [Beyene et al., 2014], Bahir Dar [Yemane et al., 2014], Hawassa [Getamesay et al., 2014] and Butajira [Mengistu et al., 2014]. Non-resistance of salmonella isolates from this study for Ciprofloxacin and Gentamycin is similar with reports from Jimma [Beyene et al., 2014] and Hawassa [Getamesay et al., 2014], but extremely lower than for Gentamycin from study done in Addis Ababa - Jimma [Beyene et al., 2011].

In this study, a high frequency of other bacterial species antimicrobial resistance to commonly used antibiotics such as ampicillin (100%), Amoxicillin (80%), Cotrimoxazole (60%), Tetracycline (45.8%), Chloramphenicol (40%) and Nalidixic acid (20%) were observed (Table 6). This finding cannot be discussed against previously reported studies on children until confirmed with antisera or PCR methods.

One of the limitations of this study was that due to lack of resource and facilities, it was not possible to conduct identification of strain groups (other bacterial isolates) and other susceptibility test methods which would show us other strain distribution and antibiotic profile at the site.

Conclusion and recommendation: This study suggests that *Shigellia*, *Salmonella* and other enteric bacteria species are some of the most pathogenic infection among children with diarrhea aged fewer than 5 years old examined in Ambo Town Public Health Intuitions. Those pathogens and enteric bacteria were found in association with mucoid diarrhea and bloody diarrhea. In addition to 9 confirmed pathogenic bacteria (6 *Shigellia* and to 3 *Salmonella* spp) there were 15 other bacteria species which cause childhood diarrhea which showed high antibiotic resistance. The highest prevalence of antimicrobial resistance was to ampicillin followed by Tetracycline and Amoxicillin. Though still at low levels, the major concern from this finding is the emerging resistance of enteric pathogens that was observed to Nalidixic acid and Cefotaxime. Multiple antimicrobial resistances were high among the isolated bacteria. Hence government should consider this serious problem of antimicrobial resistance issue by controlling black market. Ministry of health and media should also work on awareness creation on the miss use of antibiotics. Institutions should have information desk on drug adherence to minimize the resistant pattern. In general further study is also important to determine common enteric pathogens that cause diarrhea by using further laboratory methods to monitor there resistant pattern.

5. References

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6. Appendix

ANNEXE I (questionnaires)

Addis Ababa University

Collage of Health Science

Master of Medical Microbiology Program

Questionnaire

CUFY	Date ____/____/____	Code _____	
Diarrhea	Status		
	<input type="checkbox"/> Yes	<input type="checkbox"/> No	
Address			
Date of Birth	____/____/____	____/____/____	
Age Category	<input type="checkbox"/> < 2	<input type="checkbox"/> 2-5	
Gender	<input type="checkbox"/> male	<input type="checkbox"/> female	
Number of household member	Total: /_____/		
	No. <5 years old: /_____/		
Number of rooms in house	/_____/		
No. of household workers	<input type="checkbox"/> none	<input type="checkbox"/> ≥1	
Domestic animals in the house	Poultry	<input type="checkbox"/> yes	<input type="checkbox"/> no
	Goat	<input type="checkbox"/> yes	<input type="checkbox"/> no
	Sheep	<input type="checkbox"/> yes	<input type="checkbox"/> no
	Cattle	<input type="checkbox"/> yes	<input type="checkbox"/> no
	Donkey	<input type="checkbox"/> yes	<input type="checkbox"/> no
Drinking water	Tap water		

	Filtered water	
	Boiled water	
	Bottle water	
	Domestic well	
	Other	
Type of diarrhea		
Duration of diarrhea (days)	<input type="checkbox"/> < 24h <input type="checkbox"/> other: / _____ / days	
No. of stools in the last 24h	/ _____ / per day	
No. of household members reported diarrhea within 10 days before patient's illness	/ _____ /	
Received antibiotic within 4 weeks before the beginning of the diarrhea	<input type="checkbox"/> yes <input type="checkbox"/> no <input type="checkbox"/> name of antibiotic: / _____ /	
Fever at attendance time (please check the temperature by measuring)		
Chills during diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
Vomiting during diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
Bloody diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
Mucous diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
Loss of weight	<input type="checkbox"/> yes	<input type="checkbox"/> no
Stool examination	<input type="checkbox"/> yes	<input type="checkbox"/> no
Antibiotic therapy for diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
	If yes: Name of antibiotic: / _____ / Duration of antibiotic therapy: / _____ / days	
Persistence of diarrhea	<input type="checkbox"/> yes	<input type="checkbox"/> no
Microscopic examinations result		
Stool culture results		
Positive	<input type="checkbox"/> yes <input type="checkbox"/> no <input type="checkbox"/> Not performed	

culture	<input type="checkbox"/> <i>Salmonella spp</i> <input type="checkbox"/> <i>Shigella spp</i> <input type="checkbox"/> <i>Vibrio spp</i> <input type="checkbox"/> Other: / _____ /
---------	---

Thank you for your cooperation

Principal Investigator

Wagi Tosisa

Supervisor

አዲስ አበባ የንግድ ባንክ

ጤና ማረጋገጫ ሰነድ

የማስተርና ስድስት ወር ስራ ለማስፈጸም የሚያስፈልገውን ጥናት መጠይቅ

ከ5 ዓመት ዕድሜ በታች ሕፃን	ቀን _____ / _____ / _____	መለያ _____
ተቆማ ጥ	ሁኔታ	
	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>
አድራሻ _____		
የትውልድ ዘመን _____ / _____ / _____		
የዕድሜ ወሰን < 2 ዓመት <input type="checkbox"/>	2 - 5 ዓመት <input type="checkbox"/>	
የታወቀ <input type="checkbox"/>	ሴት <input type="checkbox"/>	
የቤተሰብ ብዛት ጠቅላላ / _____		
ከአምስት ዕድሜ በታች ብዛት _____		
የመኖሪያ ክፍለ-ሎች ብዛት _____		
ሥራ ያላቸው የቤተሰብ ብዛት ≥ 1 <input type="checkbox"/> የለም <input type="checkbox"/>		
የቤት እንስሳት በማኖሪያ በቤት ውስጥ	ዶሮ	አለ የለ <input type="checkbox"/> <input type="checkbox"/>
	ፍየል	አለ የለ <input type="checkbox"/> <input type="checkbox"/>
	በግ	አለ የለ <input type="checkbox"/> <input type="checkbox"/>
	የቀንድኩብት	አለ የለ <input type="checkbox"/> <input type="checkbox"/>
	አሀያ	አለ የለ <input type="checkbox"/> <input type="checkbox"/>
የመጠጥ ወሃ	ቧንቧ	
	የተጣራ	
	ፈልጎ የቀዘቀዘ	
	የተሸግ	

	የጉድጓድዉሃ		
	የወንዝዉሃ		
	ሌላ		
የተቅማጥዓይነት			
ተቅማጥ-የቆያበትጊዜ (ቀናት)	< 24 ሰዓት <input type="checkbox"/> ሌላ <input type="checkbox"/>		
በ24 ሰዓትዉስጥሰንትጊዜአስቀመጣት (ዉ) _____			
ባለፉት 10 ቀናትበቤትዉስጥበተቅማጥየተያዘየቤተሰብብዛት			
ተቅማጥበመከሰቱከ4 ሳምንትበፊትየወሰዱትመድኃኒትአንቲባዮትክስ (antibiotics) አዎ <input type="checkbox"/> አዎከሆነየመድኃኒቱዓይነት			
አይደለም <input type="checkbox"/>			
ትኩሰትአለ (መለካት)		
ብርድብርድማለትአለ	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ትዉከት	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ደምየቀላቀለተቅመት	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ንፍጥነገርአለ	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ክብደትቀንሶዋል	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ለተቅማጥ-የሰገራምርመራአድርጎል	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ለተቅማጥ-መድኃኒወስዶዋል	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
	አዎከሆነየመድኃኒቱስም		
	ስንትቀን		
ተሸሎታል	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
ተቅማጥ-ቀጥለዋል	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	
የማይክሮስኮፖዉጤት			
የሰገራካልቸርዉጤት			
ፖዜትፊ	አዎ <input type="checkbox"/>	አይደለም <input type="checkbox"/>	አልተሰራም <input type="checkbox"/>
	አዎከሆነ		

	ሳልሞነላዝሪያ	<input type="checkbox"/>
	ሺገላዝሪያ	<input type="checkbox"/>
	የኮሌራዝሪያ	<input type="checkbox"/>
	ሌላም <input type="checkbox"/>	

ለትብብር እጅግ አድርገን እና ማሰግናለን።

ተመራማሪ

አቶ ወጊቶስሳ

ተቆጣጣሪ

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ANNEXE II consent form

INFORMATION SHEET AND CONSENT FORM (ENGLISH VERSION)

General Information

Hello! How are you? My name is _____, I am working as a data collector for the study being conducted in this hospital by Wagi Tosisa who is studying for his master's degree at Addis Abeba University, college of health science. I kindly request you to lend me your attention to explain you about the study and being selected as the study participant.

The title of the study

Prevalence and antibiotic resistance of enteric bacterial pathogens isolated from childhood diarrhea under age five years in Ambo Town Public Health Institutions.

Purpose of the study

The main aim of this research is to write a thesis as partial fulfillment of a Master's degree in Medical microbiology for the principal investigator and to provide basic information about prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhea less than five years in Ambo public health institution. The data may be aid for West Showa Zone Health Bureau to develop plan for prevention and management of this disease.

Procedure and duration

Specifically I am going to ask you for information about prevalence and antibiotic resistance of bacterial pathogens among childhood diarrhea. I kindly requesting that you will advise your child to give about half of tea spoon in order to determine the prevalence of this infection. The completion of the questionnaire will take about 20 minutes, so I kindly request you to give me the sample of stool and spare me this time for filling this questionnaire. I would greatly appreciate your help.

Risks and benefits

The risk of participating in this study is very minimal, but only taking few minutes from your time. There would not be any direct payment in participating in this study, but the correct

information that you provide have a greater importance in modifying and improving the service that are provided for the clients and in identification antibiotic resistance of pathogenic bacteria.

Confidentiality

I strongly assure that your name and other identifier of your status will not be documented in the questionnaires and the information and sample you provide will be kept confidential and will not be used for anything other than the research purpose. The findings of this study will be general for the study community and will not reflect anything particular of individual persons. The questionnaire will be coded to exclude showing names and the sample is only accessible to the principal investigator.

Rights

Participation for this study is fully voluntary. You are not forced to advise your child and you have the right to decline at any time in between and this will not label your child for any loss of benefits which she/he otherwise is entitled. You do not have to answer any question that you do not want to answer. You can ask any questions at any time.

Contact address

If there are any questions or enquires any time about the study or the procedures, please contact:

Name and address of the principal investigator: Wagi Tosisa, Addis Ababa University, Tel. 0911894201 mailing address, wagitosisa@yahoo.com

Contact address of the responsible Institutional Ethics Review Committee (IRERC) at office, Addis Ababa University Health Science College

Tel _____

ANNEXEIII: DECLARATION OF INFORMED VOLUNTARY CONSENT

I am informed the participant of my child information sheet. I have clearly understood the purpose of the research, the risks and benefits, issues of confidentiality, the rights of participating and the contact address for any queries. I have been given the opportunity to ask questions for things that may have been unclear. In addition, I have been informed that I have the right to not participate my child and decline at any time. Based on the information, I confirm my agreement to participate my child on the study and advice to provide the sample and necessary information with my signature as indicated below.

Signature of the child family _____ signature of the data collector _____

ANNEXEIV: SEROLOGICAL TESTS FOR *SALMONELLA* AND *SHIGELLA* SPECIES

Test procedure of slide agglutination for *Salmonella* species by polyvalent O antisera was done by following the 3 steps of manufacturer instruction to confirm its genus.

1. Put two separate drops (40 µl each) of 0.85% saline on a glass slide. Emulsify portions of the culture under test with a loop in each drop of 0.85% saline to give a smooth, fairly dense suspension.
2. To one suspension, as a control, add one drop (40 µl) of 0.85% saline and mix. To the other suspension add one drop (40 µl) of undiluted antiserum and mix
3. Rock the slide gently for one minute and observe for agglutination using indirect lighting over a dark background. Discard the used slide for safe disinfection and disposal.

Interpretation: Slide agglutination should be strong and clearly visible within one minute. There should be no visible agglutination in the saline control; if agglutination is seen in the control, the suspension is not suitable for testing by this method.

Tests procedure for *Shigella* groups A, B, C, and D antisera

- If agglutination occurs with group A, report: “*Shigella dysenteriae*”. Test with *S. dysenteriae* type 1 antiserum. If positive, report: “*S. dysenteriae* type 1”.
- If agglutination occurs with group B, report: “*Shigella flexneri*”.
- If agglutination occurs with group C, report: “*Shigella boydii*”.
- If agglutination occurs with group D, report: “*Shigella sonnei*”.

Annex -V: Declaration Sheet

I, the undersigned, declared that this MSc research thesis is my original work. It has not been for a degree in any other university. False statements cause the invalidation of this research thesis and may lead to other administrative or legal action.

Principal investigator

Wogi Tosisa(BSc) Address: wariwagi@gmail.comsignature:_____

Advisors

Tamrat Abebe (MSc, PhD) Address: AAU, College of Health Science, Department of Microbiology, Immunology and Parasitology Email:tamrat.abebe@aau.edu.et

Signature:_____

Adane Mihret (DVM, MSc, PhD) Address: AAU, College of Health Science, Department of Microbiology, Immunology and Parasitology adane_mihret@yahoo.com

Signature:_____