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Antibiotic resistance studies of *Escherichia coli* and *Salmonella* species isolated from diarrheal patients

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Abstract

Antibiotic resistance is a widespread problem. However, limited studies are available on *Escherichia coli* and *Salmonella* spp. associated with stool samples of diarrhea patients in Leda Diarrhea Treatment Center (DTC), Teknaf, Cox, Bazar. In this study, 51 stool samples were collected between October and December 2020 from diarrhea patients from a selected DTC. Bacteria isolation and antibiotics susceptibility testing were carried out using standard microbiological procedures. Isolates of bacteria that were resistant to more than three classes of antibiotics were considered Multidrug Resistance (MDR). About 20 bacteria that include *E. coli* (n = 15) and *Salmonella* spp. (n = 5) were isolated while the highest prevalence of 73.3% and 60% respectively were observed among the under-5 years, age group. The highest (60%) frequency of isolation of *Salmonella* spp. was found among illiterate patients. The high resistance to each of amoxicillin (80%), ampicillin (100%), and tetracycline (73.3%) among *E. coli* isolates was observed while tetracycline was the most frequently used antibiotic for diarrhea treatment among sampled patients. Among *Salmonella* spp. 100% resistance to each of amoxicillin and ampicillin was observed, while 60% resistance to tetracycline was noticed. Two and one MDR E. coli and *Salmonella* spp. were recognized respectively. The occurrence of both *E. coli* and *Salmonella* among infants and aged adults, attached with MDR characteristics presented by bacteria from this study are of public health importance. Therefore, widespread studies are necessary for the determination of the molecular epidemiology of these resistant bacteria for public health surveillance.

Keywords: *Antibiotic resistant, Multidrug resistant, Drugs sensitivity, Diarrhea, Stool samples, Escherichia coli, Salmonella spp.*

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1. Introduction

Diarrhea is defined as a disease condition characterized with the passage of three or more loose stools per day or more frequent passage than is normal for a healthy individual. Diarrheal disease is a leading cause of mortality and morbidity across the globe. WHO reported increased episode of diarrhea every year in developing countries. Diarrheal disease affects all age groups, infants and children below the age of five and these age groups are more susceptible to diarrheal diseases than any other age groups (WHO, 2013; and Troeger *et al*. 2017). As at the end of 2019, Bangladesh still placed second among the top 15 countries with high child mortality due to diarrhea and pneumonia (Billah *et al*., 2019). Diarrhea is an infection of intestinal tract characterized with gastroenteritis, stomach cramps, vomiting, and dehydration which can be triggered by a variety of bacterial, viral and parasitic organisms. Infective bacterial diarrhea is a global health problem. Numerous bacterial species have been associated as a cause of infective diarrhea as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *Vibrio* spp., *Yersinia* spp., and Shiga toxin producing *E. coli* O157:H7 strain are progressively being reported as causes of acute diarrhea (Sánchez and Holmgren, 2005).

E. coli is a rod-shaped Gram-negative facultative anaerobic bacterium that belong to the family Enterobacteriaceae. *E. coli* strains implicated in diarrhea are referred to as diarrheagenic *E. coli* (DEC). DEC are one of the most important of the various etiological agents of diarrhea, which includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), as well as, diffusely adherent *E. coli* (DAEC) among others. These pathotypes differ regarding their preferential host colonization sites, virulence mechanisms, and the ensuing clinical symptoms and consequences, and are identified using molecular techniques including primers that is specific to each pathotypes (Adesoji and Liadi, 2020). *Salmonella* a rod shaped, Gram-negative bacteria of the family Enterobacteriaceae, causes a wide range of human diseases, such as enteric fever, gastroenteritis, endocarditis, and bacteraemia. It constitutes a major public health burden and exemplifies a significant health cost in many countries. Non-typhoidal *Salmonella* (NTS) such *S. typhi*, *S. paratyphi*, *S. enteritidis* and *S. choleraesuis* among others are the major cause of diarrhea (Akinyemi *et al*., 2007). Antibiotic resistance pattern demonstrated by diarrheagenic *E. coli* strains and *Salmonella* spp. are quite uniquely linked with the presence of some specific antibiotic resistance genes such genes that encode resistance against tetracycline (*tetA* and *tetB*), ampicillin (*CITM*), and chloramphenicol (*cat1* and *cmlA*) among others. Antibiotic resistant diarrheagenic *E. coli* and *Salmonella* species cause severe diarrheal disease, and could be associated with treatment failures among diarrheal patients resulting in increasing mortality associated with infective bacterial diarrhea (WHO, 2005).

Diarrheal diseases remain among the most common causes of mortality and morbidity particularly in developing countries such as Bangladesh. Risk factors associated to scanty of water, sanitation, poor personal hygiene, and malnutrition remain excessively high. Consumption of tainted food and water with pathogenic (*E. coli* and *Salmonella* spp.) can lead to infective bacterial diarrhea. Antibiotic resistance is a worldwide public health concern, particularly in settings where few treatment options are available in the treatment of infective bacterial diarrhea. However, there is paucity of information on the antibiotic studies of *E. coli* and *Salmonella* spp. linked with diarrheic stools of patients in Leda Diarrhea Treatment Center (DTC), Teknaf, Cox's Bazar, which makes it imperative to carry out this study. This research is, therefore, aimed at determination of antibiotic of *E. coli* and *Salmonella* spp. associated with stool samples of diarrhea patients attending Leda DTC, Teknaf, Cox's Bazar.

2. Materials and methods

This study was conducted in Leda DTC, Teknaf, Cox's Bazar, Bangladesh. It is located in the southern part of Bangladesh. The inhabitants are generally relay on fishing and salt farming. 51 diarrheic stool samples were collected between October and December, 2020 in the Cary Blair Transport media from diarrheal patients at Leda DTC, and transported on ice packed with the maintaining appropriate temperature to the laboratory of Department of microbiology, SAIC College of Medical Science and Technology. Semi-structured questionnaires was administered to patients or care giver of children who participated in this study for collection of demographic information, antibiotics and medications used in the treatment of diarrhea, with patients categorical classification (Both in patients and out patients) that might influence laboratory results obtained.

2.1. Ethical approval

Ethical approval was obtained from Institutional Review Board of SAIC College of Medical Science and Technology and informed verbal and written consent was obtained from patients, as well as parents or guardians of the children that were sampled.

2.2. Isolation of bacteria

A culture media enriched with brain-heart infusion (BHI) broth was used to support the likely growth of pathogens. Following the inoculation, the media was incubated and sub-cultured into *Salmonella-Shigella* agar and Mac-Conkey agar; subsequently the plates was incubated at 37 °C for 24 h in an incubator. Afterwards, the plate was observed for colony formation after 24-48 h of incubation. Distinct green metallic sheen colonies and pink colonies was aseptically picked, streaked and stored on Nutrient agar slant for further biochemical characterization. Colonial appearance such as size, shape, color, elevation, and differential characteristics such as pigmentation, lactose fermentation on MacConkey agar and Gram staining were carried out to further identify the isolates. Gram-staining and conventional biochemical methods were used to identify the *Salmonella* isolates. A culture media enriched with BHI broth was used to support the likely growth of pathogens. Following the inoculation, the media was incubated and sub-cultured into *Salmonella-Shigella* agar, blood agar, and Mac-Conkey agar. Triple Sugar Iron (TSI) agar was used initially to differentiate the isolated *Salmonella* strains, resulting in alkaline slant, acidic butt and H_2S production. *E. coli* and *Salmonella* species isolated were subjected to various biochemical tests such as: gram-stain, motility test, urease test, indole test, methyl-red, vogues proskauer test, citrate test, oxidative fermentation test, TSI agar test for biochemical characterization, and identified according to the method of Cowan and Steel (1994). Finally, *E. coli* and *Salmonella* species isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disc-diffusion method was performed on Muller-Hinton agar plates. A bacterial lawn was prepared by transferring overnight grown bacteria colonies to a glass tube containing 5 ml sterile normal saline water with a sterile inoculating loop. The suspension was vortexed and visually matched with 0.5 MacFarland standard for turbidity. Sterile cotton tipped swab was immersed in the suspension, excess fluid was removed by rolling the swab on the upper part of the tube, and spread onto Mueller Hinton agar (Oxoid, UK) to obtain a semi-confluent growth. Disks impregnated with predetermined amounts of antibiotics was dispensed onto the bacterial lawn and the plates was incubated for 18-24 h at 37 °C. After the incubation, the diameter of the inhibition zones was measured in millimeters using ruler and interpreted as sensitive, intermediate, and resistant using the criteria described by the Clinical and Laboratory Standards Institute (Cockerill *et al*., 2012). These antibiotics are used in humans for treatment of gram negative pathogens (Table 1).

Table 1: Antibiotics used in the study

Antibiotics	Types
Chloramphenicol (30 μg)	Trimethoprim / sulfamethoxazole
Amoxicillin (10 μìg)	Combination of amoxicillin, a β-lactam antibiotic and potassium clavulanate, a β-lactamase inhibitor
Ampicillin (10 μg)	Penicillin
Ceftriaxone (30 μg)	Cephalosporins of 2nd generation
Ciprofloxacin (10 μg)	Fluoroquinolone of 2nd generation
Nalidixic acid (30 μg),	Third-generation cephalosporin
Imipenem (10 μg)	Cephalosporins of 3rd generation
Streptomycin (10 μg)	Third-generation cephalosporin
Tetracycline (30 μg)	Glycylcyclines of 3rd generation

3. Results

In this study, a total of (39.21%) bacteria isolates comprising of (29.42%) *E. coli* and (9.80%) *Salmonella* spp. were isolated among (100%) diarrheic stools samples (Table 2). The percentage occurrence of bacteria isolate was higher in female (55%) than their male (45%) counterpart. It was also observed that (60.79%) stools samples showed no bacterial growth (Table 2). It was observed that *E. coli* and *Salmonella* spp. were commonly isolated among patient aged 0-5 years and age distribution ≥60 age group. However, only *E. coli* was isolated among the age distribution of ≤19 age group. While no *E. coli* and *Salmonella* spp. were isolated among patient within the age distribution of 20-39 age group and 40-59 age group (Table 3).

Table 2: Distribution of isolated bacterial among the study population (*n* = 51)

Bacteria	No. of samples positive	(%)	No. of samples negative	(%)	Total sampled	(%)	Sex					
							Male positive	(%)	Female positive	(%)	Total	(%)
Escherichia coli	15	(29.42%)	36	(70.58%)	51	(100%)	7	(46.7%)	8	(53.3%)	15	(100%)
Salmonella spp.	5	(9.80%)	46	(90.20%)	51	(100%)	2	(40%)	3	(60%)	5	(100%)

Table 3: Age and gender distribution of *Escherichia coli* and *Salmonella* spp. isolates.

Age group (years)	*Escherichia coli*			*Salmonella* spp.		
	Male	Female	Total (%)	Male	Female	Total (%)
0-5	6	5	11 (73.3%)	2	1	3 (60%)
≤19	1	2	3 (20%)	0	0	0 (0%)
20-39	0	0	0 (0%)	0	0	0 (0%)
40-59	0	0	0 (0%)	0	0	0 (0%)
≥60	0	1	1 (6.7%)	0	2	2 (40%)
Total	**7**	**8**	**15 (100%)**	**2**	**3**	**5 (100%)**

It shows male patients as the highest (58.82%) with diarrhea symptom compared to their female counterpart (41.18%) in this study. To limit bias no patient was omitted except a few patients who denied to participate. A total of 36 (70.59%) outpatients participated in the study while 15 (29.41%) were in patients. The frequency of isolation for *Salmonella* spp. was higher (60%) among in-patient than outpatients (40%). The frequency of isolation for *Salmonella* spp. was also higher (60%) among illiterate than any other group in the study participants. Antibiotics (50.98%), remain the sole treatment option reflected in treatment of diarrhea among the study participants, followed by zinc tablet supplement (31.37%) and least considered treatment option is the anti-diarrheal agent (17.65%) (Table 4). All the fifteen (15) isolates of *E. coli* were susceptible to chloramphenicol (100%) while highest frequency of resistance was detected to ampicillin (100%) follow by amoxicillin (80%), while percentage resistance to tetracycline and imipenem was observed to be 73.3% and 46.7% respectively (Table 5). Furthermore, all the 5 *Salmonella* spp. isolates were vulnerable to each of chloramphenicol and ciprofloxacin (100%) while 80 and 60% were susceptible to each of nalidixic acid and streptomycin respectively. In addition, *Salmonella* spp. exposed (100%) resistance to each of ampicillin and amoxicillin, followed by (60%) to tetracycline and (40%) to imipenem.

Multidrug Resistance (MDR) bacteria were selected based on resistance to over three classes of antibiotics. Two (2) *E. coli* isolates displayed MDR characteristics as they were resistance to more than 3 different classes of antibiotics which include aminoglycosides, cephalosporins, fluoroquinolones, penicillins, quinolones and tetracyclines. However, only one *Salmomella* spp. showed MDR characteristics as they were resistant to 3 different classes of antibiotics which include aminoglycosides, penicillins, quinolones and tetracyclines. One isolate of both *E. coli* and *Salmonella* spp. was found resistant to 3 similar antibiotics which include tetracycline,

Table 4: Sociodemographic distribution of bacteria isolates of the study participants

		Frequency number N (%)	*Escherichia coli* N (%)	*Salmonella* spp. N (%)
Total		**51 (100%)**	**15 (100%)**	**5 (100%)**
Gender	Male	30 (58.82)	7 (46.7)	2 (40)
	Female	21 (41.18)	8 (53.3)	3 (60)
Severity	In patient	15 (29.41)	5 (33.3)	3 (60)
	Out patient	36 (70.59)	10 (66.7)	2 (40)
Education level	Illiterate	24 (46.3)	8 (53.3)	3 (60)
	Basic 1-6	10 (19.5)	3 (20)	1 (20)
	Jss1-Sss3	12 (24.4)	4 (26.7)	1 (20)
	Undergraduate	5 (9.8)	0 (0)	0 (0)
Medication	Zinc tablet and ORS	16 (31.37)	0 (0)	0 (0)
	Antibiotics	26 (50.98)	0 (0)	0 (0)
	Anti-diarrheal agent	9 (17.65)	0 (0)	0 (0)

Table 5: Antibiotic susceptibility pattern of *Escherichia coli* and *Salmonella* spp. isolates

Antimicrobial agents	Number (%) Resistant (R)	Number (%) Intermediate (I)	Number (%) Sensitive (S)
***Escherichia coli* (*n* = 15)**			
Amoxicillin	12 (80)	1 (6.7)	2 (13.3)
Ampicillin	15 (100)	0 (0)	0 (0)
Ceftriaxone	5 (33.3)	4 (26.7)	6 (40)
Chloramphenicol	0 (0)	0 (0)	15 (100)
Ciprofloxacin	1 (6.7)	3 (20)	11 (73.3)
Imipenem	7 (46.7)	2 (13.3)	6 (40)
Nalidixic acid	1 (6.7)	3 (20)	11 (73.3)
Streptomycin	3 (20)	1 (6.7)	11 (73.3)
Tetracycline	11 (73.3)	3 (20)	1 (6.7)
***Salmonella* spp. (*n* = 5)**			
Amoxicillin	5 (100)	0 (0)	0 (0)
Ampicillin	5 (100)	0 (0)	0 (0)
Ceftriaxone	1 (20)	1 (20)	3 (60)
Chloramphenicol	0 (0)	0 (0)	5 (100)
Ciprofloxacin	0 (0)	0 (0)	5 (100)
Imipenem	2 (40)	3 (60)	0 (0)
Nalidixic acid	1 (20)	0 (0)	4 (80)
Streptomycin	1 (20)	1 (20)	3 (60)
Tetracycline	3 (60)	1 (20)	1 (20)

nalidixic acid, and streptomycin (Table 6). Figure 1 shows routinely, commonly used antibiotics among the study participants which may have contributed to the vast increase in antibiotics resistant among the bacteria, emergence of MDR that may lead to treatment failure, antibiotic mediated diarrhea and false negative bacterial culture from patient sampled. Majority of the study participants lack basic knowledge on the name and meaning of antibiotics. In addition tetracycline and metronidazole remain the most commonly used antibiotics in the treatment of diarrhea among the patient sampled in the study participants.

Table 6: Multidrug resistant profile of bacteria isolates

Microorganisms	No. of MDR	Resistant pattern
Escherichia coli	Two (2)	CEFT, STREP, TET, AML, AMP, AMP, AML, CEFT, CIPX, NA
Salmonella spp.	One (1)	NA, AML, AMP, STREP, TET

Note: KEYS; AMP = Ampicillin, AML = Amoxicillin, CEFT = Ceftriaxone, CIPX = Ciprofloxacin, NA = Nalidixic acid, STREP = Streptomycin, TET = Tetracycline, and MDR = Multi Drug Resistant profile.

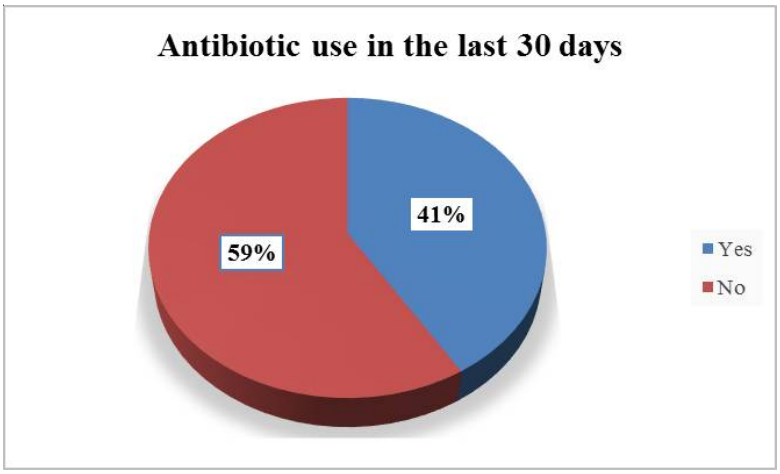

Figure 1: Distribution of antibiotics taken by the study participants in the last 30 days before hospital admission

4. Discussion

In this study, 29.42% occurrence of *E. coli* was observed among the study participants (Table 2), which is lower compared to 41.4% occurrence of *E. coli* reported by Korie *et al*. (2012) and 59% occurrence of *E. coli* from diarrheic stools reported by Dormanesh *et al*. (2015) in a study conducted in Nigeria and Iran respectively. Kalule *et al*. (2018) reported 4.5% occurrence of *E. coli* from diarrheic stools in a study conducted in South Africa. However, 9.80% occurrence of *Salmonella* spp. was detected in this present study, which is higher than 1.2% and 5% occurrence of *Salmonella* spp. reported by Nair *et al*. (2015) and Mzungu *et al*. (2016) in a study conducted in India. Lamboro *et al*. (2016) reported 10.8% occurrence of *Salmonella* spp from diarrheic stools in a study conducted in Ethopia, Akinyemi KO *et al*. (2007) [16] described 17% occurrence of *Salmonella* spp. from stool samples of patients with gastroenteritis in a study conducted in Nigeria. This inconsistency may be attributable to differences in the study designs, patient's selection, differing environmental condition and behavioral pattern in those regions.

In this study the highest frequency of bacterial isolation from diarrheic stools where among the subjects in the age group under-5 years (Table 3) and this is study similar to reports by other authors from Nigeria, Senegal and Saudi Arabia (Kalule *et al*. 2018; Thiam *et al*. 2017; and Al-Jurayyan, 1994). This could be because diarrhea could result from hand contamination among this children especially while playing on the ground,

playing with toys or other objects, and accidentally putting their dirty finger in their mouth. In addition, the risk of ingesting tainted materials among this age group is extremely high, especially in unhealthy environments. The low frequency of isolation among young adult within the age group of ≤19 might be related with the development of immunity or loss of receptor for specific adhesion molecules. Similarly, stools positive culture for bacteria observed among seniors within the the age category of ≥60 might be associated with the fact that surveys on seniors have found that non negligible proportion of elder adult do not follow recommended food safety practices, which makes them vulnerable to gastroenteritis linked with the studied bacteria, i.e., *Salmonella* spp. There was higher frequency of stool culture positive for bacteria among male within uder-5 years than their female counterpart. This may be due to the fact that males are more generally active than females and therefore more prospective to pick particles from the ground and put into their mouth, while females in this part of the country are more protected than the males. Diarrheic stools samples from females within the age category of 19 and 59 tested positive for bacterial culture than their male counterpart. This may be elucidated by the fact that adult females are closer to young children that are more susceptible to infective bacterial gastroenteritis which can be moved through person to person by contact with infected individuals.

This study indicated that *E. coli* isolates presented high resistance rate to ampicillin (Table 5). This findings is in agreement with reports from Thailand and Kenya (Wilunda and Panza, 2009; and Sang *et al*., 2011). The antibiotics sensitivity pattern shows high sensitivity to ciprofloxacin and chloramphenicol which is similar to study conducted in Ethopia and Oman (Sang *et al*., 2011; Adugna *et al*., 2015; and Ali *et al*., 2010). In this present study, the 5 *Salmonella* spp. isolates showed 100% sensitivity to chloramphenicol and ciprofloxacin in line with the report from a study conducted in Ethopia (Beyene and Tasew, 2014). These increase in resistance may be attributed to the widespread misuse of this drug, coupled with the fact that they are cheap, people can purchase these drugs from the open market in the study area without physician prescription. Increased and high susceptibility to chloramphenicol might be attributed to the cost of the antibiotics, it may be expensive compared to ampicillin, amoxicillin and tetracycline which cannot be easily afforded by majority of the people from the study area, and therefore this may have contributed to the effectiveness of the chemotherapeutic agent in treatment of diarrhea caused by *Salmonella* spp.

High resistance of bacteria (*E. coli* and *Salmonella* spp.) to beta lactam antibiotics observed in this study (Table 5) is also in agreement with high resistance of bacteria to beta lactam antibiotics reported by Adesoji and Ogunjobi (2016) from non-clinical samples. In addition, occurrence of tetracycline and streptomycin resistance among MDR bacteria (*E. coli* and *Salmonella* spp.) observed in this study (Table 6) is similar to tetracycline resistant MDR and aminoglycoside resistant MDR bacteria reported by Adesoji *et al.* (2019) from environmental samples in a study (Timilehin *et al*., 2019). This might be explained by the fact that indiscriminate use of antibiotics may have contributed to vast emergence and spread of multidrug resistant bacteria from both clinical and non-clinical environment. From this present study, the frequency of isolation was highest with (60%) *Salmonella* species isolates from hospitalized patients (Table 4), this is in contrast to (5.4%) *Salmonella* species isolates from hospitalized patients reported by Thompson *et al.* (2013) in a study conducted in Vietnam. This is evident that *Salmonella* associated diarrhea can be fatal in immunological naïve patients. Likewise, in relation to educational status and frequency of isolation, this study indicated highest isolation (60%) of *Salmonella* spp. was observed among illiterates (Table 4). This result is consistent with earlier studies conducted by Lamboro *et al* . (2016). Education is vital to create awareness in the community with regard to the mechanism of management of infectious diarrhea and control of other factors that lead to this disease.

In this study, increased use of tetracycline (41.17%) indicated among the sampled patients (Figure 1) might have contributed to high percentage of resistance to tetracycline (14, 70%) observed in this study. The result of high negative stool culture (Table 2) might be as a result of pretreatment with antibiotics among the sampled patients within the last 30 days. This is similar to report by Korie *et al.* (2012) Oni *et al*. (1991). In addition this might also be due to the fact that pathogens such as parasites and viruses are also implicated in diarrheal diseases (Abdullahi *et al*., 2010). Antidiarrheal agent contain Loperamide hydrochloride sold and known to the sampled patients under the brand name of (Lemotil and diarrhea stop) which is used in the treatment of antibiotics associated diarrhea, was found to be the least treatment options considered for treatment of diarrhea in the study area (Table 4). With the vast increase of resistance to commonly used antibiotics associated with the studied bacteria, antidiarrheal agent could be considered as an effective alternative medication to antibiotics with respect to diarrhea diseases. Though, susceptibility of bacteria isolates to chloramphenicol and fluoroquinolones (ciprofloxacin) observed in this study (Table 5) displays that these chemotherapeutic agent

remains effective in treatment of *E. coli* and *Salmonella* species associated diarrhea as reported from other developing countries.

5. Conclusion

In conclusion, from this present study, *E. coli* and *Salmonella* spp. were frequently isolated among under-5 age groups with prevalence of 73.3% and 60% respectively. However, the results of antibiotic susceptibility tests in this study presented high level of resistance among isolates especially to ampicillin (100%), amoxicillin (80%) and tetracycline (70%) building them completely unpredictable in the management of *E. coli* and *Salmonella* spp. associated diarrhea in the study area. It was also observed that (15%) of bacteria displayed multi drug resistant characteristics. Hereafter, comprehensive studies are required for the determination of the molecular epidemiology of these resistant bacteria for public health surveillance.

Conflicts of interests

The authors declared no conflicts of interest.

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