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Isolation of Cefixime Resistant *Salmonella* from Hospitals waste and Profiling Multi-drug Resistance Pattern of the Selected isolates

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Abstract

Anti-microbial resistance is a serious and emerging crisis for both developed and developing countries throughout the world. Irrational and indiscriminate use of antibiotics flourishes the development of Multi-drug resistance (MDR) pathogens, raising some conjecture that we are almost at the verge of antibiotic era. Public hospitals play a major role in the evolving of MDR bacteria, because of frequent and excessive use of antibiotics is practiced here. The objectives of the study are the isolation of cefixime resistant Salmonella spp. from three hospitals waste samples in south-eastern region of Bangladesh (Chittagong) and the evaluation of the multidrug resistance patterns of the isolated samples. After TVC, 30 cefixime resistant Salmonella were isolated from the waste samples of three different hospitals (10 from each sample). Among the 30 isolates, 22 isolates were found resistant up to 500 µg/ml cefixime, 5 isolates showed resistance up to 400 µg/ml cefixime and rest of them were resistant up to 300 µg/ml cefixime. Isolates of cefixime resistant salmonella were further subjected to antibiotic sensitivity test by disc diffusion methods using five antibiotics e.g. penicillin, chloramphenicol, tetracycline, ciprofloxacin and azithromycin. Results showed that all the isolates were multi-drug resistant but all the isolates were also azithromycin sensitive. This result describes that most commercially available antibiotics are ineffective against Salmonella whereas azithromycin is still effective against Salmonella and it might be a good choice for the infections caused by Salmonella spp.

Keywords: Bangladesh, salmonellosis, multidrug resistance, susceptibility, antibiotic gastroenteritis, enteric fevers.

Introduction

Salmonellosis is one of the most widespread food-borne diseases causing a major public health burden and attributing a significant cost in both developed and developing countries. The causative agents of these maladies are the bacteria *Salmonella spp. which* may cause three types of infections namely enterocolitis (gastroenteritis), enteric fever (typhoid and paratyphoid fever) and septicemia in human[1]. The bacteria are generally communicated to human through the contaminated food consumption of animal origin such as meat, milk, poultry and eggs[2,3]. One of the recent study has estimated that nearly 93.8 million human are affected by gastroenteritis and 155 000 are died around the globe each year due to non-typhoidal *Salmonella* infections[4]. Only in the USA, for example, 1.4 million cases of non-typhoidal gastroenteritis are reported every year which leads to an estimated hospitalization of 15 000 peoples and death more than 400 individuals annually[5,6]. On the other hand, typhoid and paratyphoid fever occurred by *Salmonella enterica* serovar *Typhi* and *Paratyphi* is one of the major causes of morbidity and mortality in developing nations leading to thousands of deaths every year[7]. These enteric fevers are considered as one of the worst epidemic problems for Bangladesh as well as other countries of the Indian subcontinent, Central and South America and Africa with annual occurrence of more than 100 cases per 100 000 peoples are reported[8]. Statistics showed that 22 million of people are affected by enteric fever annually with 200 000 deaths worldwide[9]. The antimicrobials of different groups are most widely regarded as optimal therapy for the treatment of Salmonellosis. However, the emergence of antibiotics resistant, especially multidrug resistant, Salmonella *spp* has raised question about the inefficacy of therapeutics in coming future. The evolving of Salmonella *spp* resistant to many broad spectrum antibiotics such as chloramphenicol, ampicillin, streptomycin, tetracyclines, ciprofloxacin, kanamycin, cephalosporins, has already been reported in many developed and developing countries[10-14]. Attaining multidrug resistance capacity by Salmonella *spp* engenders therapeutic crisis worldwide which hurls clinicians in a quandary of choosing appropriate medication for Salmonellosis. Not only that, it also increases significantly the total cost of treatment. For example, according to the economic report of the United States Department of Agriculture (USDA)[15], the estimated yearly economic cost in USA due to MDR *Salmonella spp* infections is close to 25 billion US dollar, therefore precisely exceeding the yearly economic damage attributable to infections caused by *E. coli* ($460 million) or *Listeria monocytogenes* ($2 billion)[15].

The developing and spreading of resistance capacity in bacteria is an ecological phenomenon which stems due to indiscriminate and widespread use of use of antibiotics and their discharge into the environment Resistance may occur in *Salmonella* due to the excessive and promiscuous use of antimicrobials in the

treatment of humans, animals and the inclusion of growth-stimulating antibiotics to the fodders of breeding animals. Hospitals, especially in developing countries like Bangladesh, also contribute in a great extant in the development of multidrug resistant *Salmonella*. Different types of antibiotics are used for the treatment of different infectious diseases as well as for surgery purposes. Most of the antibiotics only metabolized partially by patients and are discharged into the hospital sewage system through feces and urine of patients. Continual Expose to un-metabolized or partially metabolized antibiotics of different microorganisms present in the hospital waste leads to the selection of resistant bacteria like *Salmonella* in the environment and subsequent spread to human by nosocominal transmission or by other ways[16]. The outbreaks of MDR *Salmonella* has been reported in Bangladesh[17], and occurrence of ciprofloxacin resistant *Salmonella* in Chittagong city has also been reported recently[18]. However, there is no data on prevalence of cefixime resistant *Salmonella spp.* in Chittagong city but there are several reports of emerging cefixime resistant *Salmonella spp.* in other parts of the world[19].

The aims of the current study are the isolation and characterization of cefixime resistant *Salmonella spp.* from the wastes of three most popular hospitals in Chittagong city, the densely populated south-east region of Bangladesh, and the comparative analysis of multidrug resistance patterns among the isolated samples. The outcome of this study unfolds the MDR patterns of Salmonella *spp.* that will provide appropriate guidelines for clinicians to choose suitable antibiotics for Salmonellosis in specific cases for the patients of this region. This data may be helpful for some other tropical countries in Southeast Asia to deal with such health burden.

Material and Methods

Sample collection: Three most popular hospitals in the Chittagong city were targeted for sample collection. Sample-1 was collected from Chattagram Maa-Shishu O General Hospital, Sample-2 was collected form Chittagong Medical Collage Hospital (CMCH) and sample-3 was collected form USTC Bangabandhu Memorial Hospital (BBMH). All the samples were collected by maintaining aseptic procedure in sterilized screw cap test tubes. Each sample was liquid hospital waste collected from the septic tank of the respective hospitals where discharge (stool, urine, etc) and medical waste of patients of the different wards like Gyniatrics, Surgery, Orthopedics, Medicine, Heart and General Wards are disposed. The patients in the words were of different age, sex and with various diseases and treated with different antibiotics.

Total Viable Count (TVC): After sample collection, all the samples were subjected to serial dilution up to 10^{-8} times with double distilled water in sterile condition. The nutrient agar (NA) plates were prepared afterwards with antibiotic (cefixime in a concentration of 50 µg/ml) and without antibiotic. Each of the samples diluted from 10^{-5} to 10^{-8} thereupon were transferred in separate petri-plates containing NA media (with or without antibiotic). After 24 hours of incubation at 37^0 C, plates with 30 to 300 bacterial colonies were reckoned. The numbers of the total bacteria in each plate were then enumerated by the multiplying the total colonies with the dilution factor. The total bacteria of all the three samples were counted by this procedure.

Isolation and Identification: Single colonies were picked up randomly by sterile tooth picks from the NA plates and patched on *Salmonella* agar plate containing 50µg/ml cefixime. In this way, total 30 *Salmonella* agar plates (10 for each sample) each containing 10 to 12 patched were prepared, where each patched representing a single colony. After incubation at 37°C for 24 hours, the colonies representing black color were suspected as *Salmonella.* Each single black –patched colony of previously cultured plate was then streaked by loop onto freshly prepared Salmonella agar plate containing 50µg/ml cefixime. About 30 plates were prepared by this way and were incubated at 37°C for 24 hours. The colonies with black center were picked up in slants and were coded according to the samples name such as S1cefR1, which means cefixime resistant isolate-1 from sample-1. The pure cultures of selected isolates were subjected to presumptive test (Triple Sugar Iron Test). Isolates representing positive results in presumptive test were studied further by morphological and biochemical test including Gram staining, Urease test (UT), Lactose fermentation test (LFT), Mannitol Fermentation test (MFT), Citrate test (CT), Oxidase test (OT), Methyl red test (MRT), Motility test (MT), Voges Proskauer (V.P) test.

Multi-drug sensitivity bioassay: Multidrug sensitivity test for the selected cefixime resistant *Salmonella* isolates were done by Kirby-Bauer disc diffusion method on Mueller Hinton Agar medium with disks containing penicillin G (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), cefixime (50 µg), azithromycin (Azithro, 10 µg) and cefixime (50 µg). The isolates representing resistance against at least two antibiotics were considered as multidrug resistant.

Growth measurement at different concentrations of antibiotics: For this experiment, each of the 30 isolates was inculcated onto nutrient agar plates containing different concentration of cefixime ranging from 50, 100, 200, 300, 400 and 500 µg/ml of the NA and incubated 37^oC for 24 hours. After incubation the bacterial growth was observed.

Results and Discussion

The total viable count of sample 1, 2 and 3 with and without cefixime (table 1) (figure- 1 to 4) showed that ,among the three sample, sample 3 had highest number of bacterial count without antibiotic Whereas TVC with cefixime was maximum for sample-2. In sample-1, 11.98% bacteria were resistant to cefixime (50 µg/ml). In sample-2, 16.92% bacteria were resistant to cefixime (50 µg/ml). In sample-3, 8.57% bacteria were resistant to cefixime (50 µg/ml) (table- 4 and figure- 2 to 4).

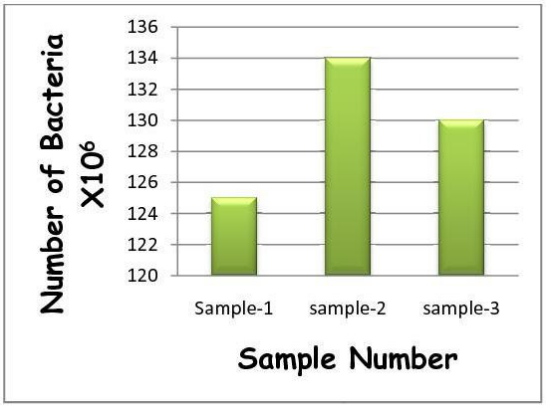

Figure-1
Total Viable Count of 3 samples

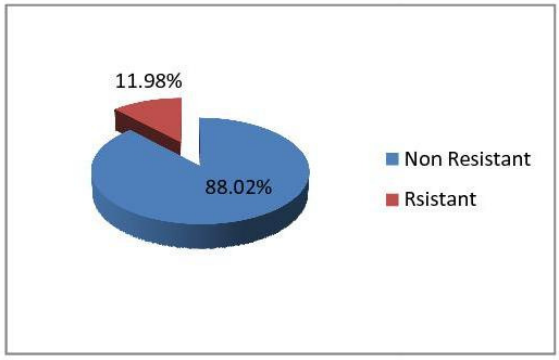

Figure-2
Percentage of Cefixime Resistant Bacteria in Sample 1

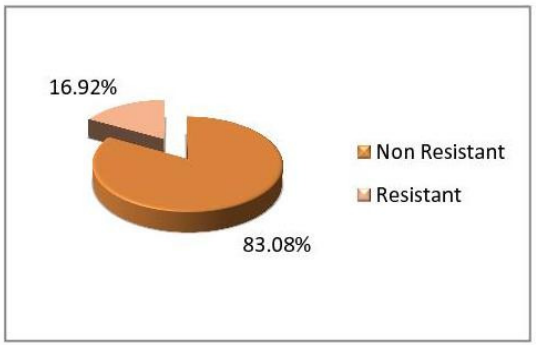

Figure-3
Percentage of Cefixime Resistant Bacteria in sample-2

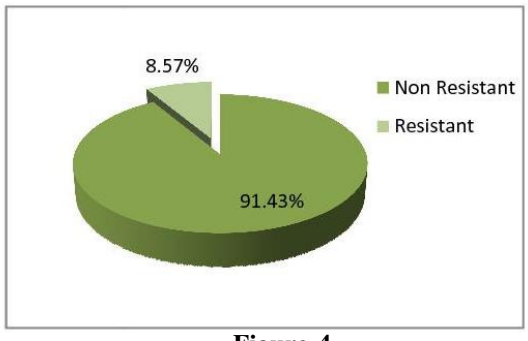

Figure-4
Percentage of Cefixime Resistant Bacteria in sample-3

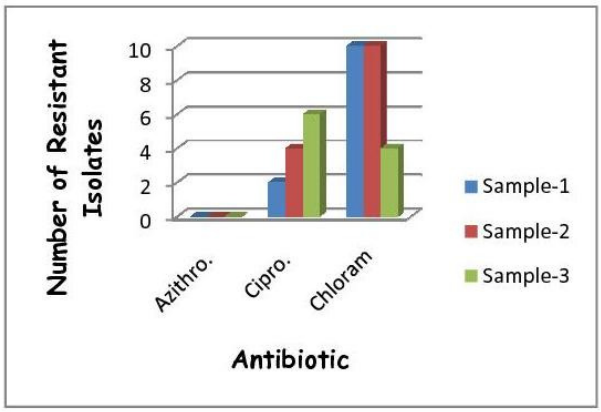

Figure-5
Comparison of antibiotic sensitivity test

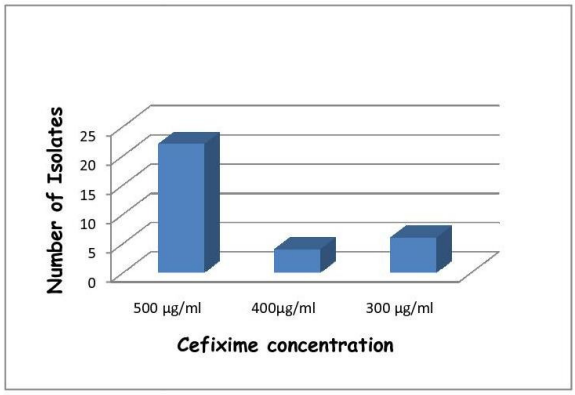

Figure-6
Level of cefixime resistance in *Salmonella* isolates

Table-1
Percentage of Cefixime resistant bacteria in sample 1, 2 and 3

Sample Number	Total Viable Count Without antibiotic	Total ViableCount With antibiotic Cefixime (50 µg/ml)	Percentage of resistant bacteria (%)
1	1.67×10^8	2×10^7	11.98
2	1.33×10^8	2.25×10^7	16.92
3	1.75×10^8	1.5×10^7	8.57

Cefixime resistant *Salmonella* spp were isolated from three hospitals waste samples using *Salmonella* agar media. Total 30 isolates (10 from each sample) were selected by presumptive test that gave positive result for *Salmonella* in TSI (Triple Sugar Iron) agar slants. This positive isolates produced alkaline slant, acidic butt, gas and H_2S in TSI agar slants. Gram staining of all the 30 isolates showed that the isolates were Gram negative and rod shaped. The isolates were confirmed further as Salmonella *spp.* by 9 bio-chemical tests (table 2). According to characteristics of the colonies, results of the presumptive tests

and the biochemical tests, the isolates were identified as Salmonella *spp.*

The result of antibiotic sensitivity assay showed that all the cefixime resistant *Salmonella* were multidrug resistant (table- 3, figure-5). Growth observation (figure-6) of isolates in different concentrations of cefixime exhibited that among the 30 isolates, 22 isolates were extremely resistant to cefixime (up to 500 µg /ml). The next 5 isolates showed resistance up to 400 µg/ml cefixime, and remaining 3 isolates had resistance capacity up to 400 µg/ml cefixime.

In this study, total number of bacteria & total number of cefixime resistant bacteria were enumerated for all samples (table 1). TVC (at 10^{-6}) without antibiotic showed almost similar result for all three samples with highest number of bacteria was found in sample- 2 and least number of bacteria was found in sample-1. The occurrence of cefixime resistant bacteria was comparatively higher in sample-2 than that of other two samples. In sample- 2, 16.92% bacteria were resistant to cefixime that was followed by sample-1(11.98%) and sample-3 (8.57%) respectively. This higher abundance of cefixime resistant bacteria in sample-2 than other samples clearly betokens that this antibiotic was prescribed frequently by clinicians in the Chittagong Medical College Hospital (CMCH).

Table-2
Summarized result of all tests

ISOLATES	Colony Character BSA	Presumptive test (TSI)				Bio-chemical test									Comment
		S	B	G	H_2S	UT	LFT	MPI	OT	CT	MT	IT	MRT	VPT	
S1CefR1	Black	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR2	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR3	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR4	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR5	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR6	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR7	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR8	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR9	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S1CefR10	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR1	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR2	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR3	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR4	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR5	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR6	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR7	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR8	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR9	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S2CefR10	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR1	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR2	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR3	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR4	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR5	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR6	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR7		k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR8	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR9	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*
S3CefR10	,,	k	A	+	+	-	-	+	-	-	+	-	+	-	*Salmonella*

BSA: Bismuth Sulphite Agar, **BGA:** Brillinat Green Agar, **S:** Slant, **B:** Butt, **G:** Gas, **H_2S:** Hydrogen sulphide, **UT:** Urease test, **LFT:** Lactose Fermentation tes, **MFT:** Mannitol Fermentation test, **OT:** Oxidase test, **CT:** Citrate test, **MT:** Motility test, **IT:** Indole test, **MRT:** Methyl red test, **VPT:** Voges Proskeaur, K: Alkaline, **A:** Acidic.

Table-3
Antibiotics sensitivity test of cefixime resistant *Salmonella* isolates from sample-1,2 and 3 against several commercial antibiotic discs

	Antibiotic sensitivity Zone diameter (mm)						Resistance
	Cef	Pen G	Chloram	Tetracyc	Cipro	Azithro	
Sample-1							
S1CefR1	0	0	7	0	25	30	MDR
S1CefR2	0	0	0	0	18	25	MDR
S1CefR3	0	0	2	5	24	34	MDR
S1CefR4	0	0	3	3	09	27	MDR
S1CefR5	0	0	0	0	22	27	MDR
S1CefR6	0	0	0	0	19	24	MDR
S1CefR7	0	0	6	4	24	29	MDR
S1CefR8	0	0	0	0	17	26	MDR
S1CefR9	0	0	0	0	11	23	MDR
S1CefR10	0	0	3	4	21	29	MDR
Sample-2							
S2CefR1	0	0	0	2	07	23	MDR
S2CefR2	0	0	0	1	15	29	MDR
S2CefR3	0	0	0	0	20	23	MDR
S2CefR4	0	0	0	6	19	31	MDR
S2CefR5	0	0	0	0	17	24	MDR
S2CefR6	0	0	0	0	21	26	MDR
S2CefR7	0	0	3	1	09	22	MDR
S2CefR8	0	0	0	0	20	25	MDR
S2CefR9	0	0	4	0	11	27	MDR
S2CefR10	0	0	0	5	20	24	MDR
Sample-3							
S3CefR1	0	1	18	4	7	34	MDR
S3CefR2	0	0	16	3	19	29	MDR
S3CefR3	0	0	12	0	18	31	MDR
S3CefR4	0	3	17	0	4	24	MDR
S3CefR5	0	0	19	0	0	25	MDR
S3CefR6	0	0	17	7	18	30	MDR
S3CefR7	0	0	24	0	3	28	MDR
S3CefR8	0	4	11	2	1	30	MDR
S3CefR9	0	0	9	3	0	26	MDR
S3CefR10	0	0	13	0	17	31	MDR

The level of cefixime resistant was remarkably high among the *Salmonella* isolates. Out of 30 *Salmonella* isolates (10 from each sample), 22 isolates were found resistant up to 500 µg/ml cefixime, 5 isolates showed resistance up to 400 µg/ml cefixime and rest of them were resistant up to 300 µg/ml cefixime. This high level of cefixime resistance in bacteria of the selected hospitals waste samples might be the consequence of excessive and irrational practice of cefixime antibiotic by physician in order to treat various diseases of patients those went through these hospitals for taking health care.

The *in vitro* antibiotics sensitivity assay of the cefixime resistant salmonella exhibited that all the *Salmonella* isolates were multidrug resistant. All the isolates of sample- 1, 2 and 3 were highly resistant to penicillin and tetracycline, indicating that these two antibiotics are no longer effective against *Salmonella* or at least *Salmonella* occurring at this locality. In contrast, all the *Salmonella* isolates were sensitive to azithromycin. This result describes that azithromycin is still effective against *Salmonella* and it might be utilized as a better choice for the infections caused by *Salmonella spp.*

The antimicrobial bioassay results for chloramphenicol and ciprofloxacin were varied substantially among the samples. All the isolates of sample-1 and sample-2 showed resistance against chloramphenical whereas, only 4 isolates (out of 10) of sample-3 represented resistance to this antibiotic. Conversely, the number of ciprofloxacin resistant isolates was highest in

sample-3 (6 out of 10), followed by sample-2 (4 out of 10). Only 2 isolates of sample-1 were found ciprofloxacin resistant. This may be due to the fact that chloramphenicol was more likely prescribed in CMCH and Chattagram Maa-Shishu O General Hospital whereas ciprofloxacin was preferably used in USTC Bangabandhu Memorial Hospital (BBMH). On the other hand, all the bacterial isolates were highly resistant to both tetracycline and penicillin. So, according to our findings, it can be inferred that tetracycline and penicillin cannot be a good therapeutic option for the treatment due to their resistance.

From the aforementioned results of antibiotic sensitivity assay, it can be overtly deduced that such high prevalence of multidrug resistant bacteria is very alarming for Bangladesh. Occurrence of such increasing patterns of multidrug resistance has also been observed in India in the last 30 year[20].

The contributing factors for the emergence of multidrug resistance capacity in bacteria of the selected hospitals wastes may be overuse or misuse of different antibiotics and improper prescribing practices by doctors coupled with plasmid mediated intrinsic microbial factors[21, 23].

The presence of un-metabolized antibiotics in the hospitals wastes may also be a dominant factor in the emergence and spread of antibiotic-resistant bacteria as similar observation was reported by *Ahmed et al.,* 2004. The most apprehensive fact of thriving multidrug resistance trait in bacteria is that the resistance attribute is transferable. Eventually, there is an immense possibility of transferring the phenotype to other bacteria of same or distantly related species, if the resistant bacteria are allowed to spread in the environment. On the other hand, there is a good chance to enter these multidrug resistant microbes into the food chain because lack of proper swage management system in the developing countries like Bangladesh. Moreover, as Bangladesh is a densely populated country, such population overload may lead to increased dispersal of multidrug resistant bacteria as well as genes which is also observed in other countries[24].

Conclusion

More than half a century has passed since the commercial introduction of first antibiotics. For microbes, it did not take long to be resistant against antibiotics. Multidrug resistance is not a problem for a particular location instead it is a worldwide concerning problem that does not obey international borders and can indiscriminately affect members of all socioeconomic classes[25]. The most alarming concerns of the emergence antimicrobial resistance in bacteria are that it may result in failure of antimicrobial therapy in coming future [26, 27].

The findings of our study show that most of the broad spectrum antibiotics are no longer effective against MDR *Salmonella spp.* However, there are still some advanced graded antibiotics such as azithromycin that retain their efficacy against the bacteria which is the consolation for us. The outcome of this study may be provided at local, national and international levels to help national guideline preparation for preventing emergence and spread of antibiotic resistant bacteria. Finally, this study suggests that physicians should be more aware during prescribing antibiotics for the treatment. In addition to this, irrational and unnecessary use of antibiotics in case of curing common flu or viral diseases might be stopped. If development of antibiotic resistance is going un-controllable, time is not so far when an effective antibiotic would not be able to treat even minor infections.

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