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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *SALMONELLA* SEROVARS FROM DIARRHOEIC STOOL SAMPLES OF HUMAN

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ABSTRACT

The present study aimed at isolation and identification of *Salmonella* serovars from human stool and characterization of the isolated serovars using biochemical, serological, molecular and antimicrobial sensitivity techniques. A total of 25 samples were collected of which 16% were positive to *Salmonella* serovars. All the culturally positive isolates fermented dextrose, maltose and mannitol with the peoduction of acid and gas but not fermented sucrose and lactose. The same isolates showed Indole and V-P tests negative but M-R test positive. All the culturally and biochemically positive *Salmonella* serovars showed agglutination with poly 'O' and poly 'H' antisera. The antimicrobial susceptibility testing showed that the isolated *Salmonella* serovars were highly sensitive to ciprofloxacin and moderately sensitive to chloramphenicol, kanamycin, cotrimoxazol and nalidixic acid. However, the positive isolates were resistant to erythromycin. The present study indicates that ciprofloxacin can be used as a first line therapy for the treatment of *Salmonella* gastroenteritis.

Keywords: Isolation, characterization, sensitivity, *Salmonella*, human stool

INTRODUCTION

Salmonellosis is a disease condition caused by a large group of bacteria of the genus *Salmonella* that can affect human being throughout the world. *Salmonella* infection remains as a serious problem to public health significance in world wide (Tubaraiae *et al.*, 1994) and causes substantial economic loss resulting from mortality, morbidity and poor growth with hazard of transmitting food poisoning with gastroenteritis to human and represents a serious problem for the food industry (Khan *et al.*, 2007). Human spreads *Salmonella* mainly through the stool. Food borne illness among the people and transmission can occur when food and water are contaminated with stool or through direct fecal-oral route. Human stool acts as an important reservoir of *Salmonella* serovars that are the grouping of micro organisms based on their cell surface antigen. Species isolated from human stool are *Salmonella typhi*, *S. paratyphi A*, *S. typhimurium*, *S. wrothington* and *S. enteritidis* (Kumar *et al.*, 2009). *Salmonella* is a world wide issue in public health sector. People most at risk for serious complications due to *Salmonella* food poisoning include older adults, pregnant women, infants, children, and people who have compromised immune systems. Salmonellosis is manifested clinically in all hosts by one of three major syndromes, per acute systemic infection, an acute enteritis or a chronic enteritis (Merchant and Packer, 1967). Symptoms are usually including headache, nausea, vomiting, fatigue, gastroenteritis, abdominal cramps and bloody diarrhea with mucus and sometimes reactive arthritis (Reiters syndrome) (Dworkin *et al.*, 2001). Following Salmonellosis dehydration with renal insufficiency and death may occur.

The importance of Salmonellosis in public health sector is a growing concern day by day throughout the world and over the last several decades there have been significant shift in predominant *Salmonella* serovars associated with human infections (Steven *et al.*, 2011). Salmonellosis in the past has caused tremendous loss to society in many countries around the world. Two to four million of cases have been reported annually and yet a significant number of cases have been unreported worldwide. Non-typhoidal *Salmonella* is the leading cause of food borne illness and its increasing antimicrobial resistance is associated with higher risks of hospitalization in Bangladesh (ICDDR,B) (Salam *et al.*, 2003). Non-typhi *Salmonella* was found responsible for 66% cases of food borne illness in Bangladesh. The highest proportion (15%) was isolated in 1998 followed by in 1995 (13%) while it was less than 10% for other years. Thirty six percent were isolated during the summer while 28% were in the fall. There is lack of sufficient studies emphasizing isolation and characterization of *Salmonella* serovars considering human stool in Bangladesh. Moreover, knowledge of manifestation of pathogenicity as well as

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antigenic mosaic of these species would certainly help in suggesting prophylactive measures there of. This, in turn, will go a long way to avoid much of our loss and inconvenience concerned in public health. In view of these considerations, the present study was undertaken with the following objectives of (i) to isolate and identify *Salmonella* serovars from human stool, (ii) to characterize the isolated *Salmonella* serovars using cultural, biochemical and serological techniques and (iii) to study the antibacterial sensitivity of the isolated *Salmonella* serovars.

MATERIALS AND METHODS

The present research was conducted during the period of June 2009 to May 2010 in the Bacteriology Laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh. A total of 25 diarrhoeic stool samples were collected for the study. Among the samples 9 were from BAU Diabetic Center (BDC) and the rest 16 were from Mymensingh Medical College Hospital (MMCH). The entire study was divided into two major steps. The first step included selection of sources, collection of samples, transportation to the laboratory, isolation and identification of Salmonellae on the basis of their colony morphology, staining property, motility and biochemical and serological characteristics. In the second step, the current statuses of drugs sensitivity and resistance patterns of isolated bacteria were determined. After collection, each sample was inoculated into freshly prepared Nutrient broth (NB), identified into tube properly and incubated at 37^0C for 24 hours aerobically in bacteriological incubator. Then, a loop full of bacterial culture from incubated tubes were streaked separately into the Salmonella-Shigella agar (SSA), McConkey agar (MCA), Eosin methylene blue agar (EMBA) and Brilliant green agar (BGA) plates. The plates were examined and studied carefully for the presence of characteristic colonies of Salmonellae. Nailidixic acid (NA) medium was used to grow the organisms from the collected samples. The representative *Salmonella* colonies were characterized morphologically using Gram's staining method according to the method described by Merchant and Packer (1967). For carbohydrate fermentation tests, triple sugar iron agar (TSIA) slant reaction, methyl red-Voges-Proskauer (MR-VP) and Indole reaction tests were carried out for identification of suspected *Salmonella* according to the methods described by Douglas *et al.* (1998) and OIE (2000). The carbohydrate fermentation test was performed by inoculating a loop full of NB culture of the organisms into the tubes containing different sugar media. Acid production was indicated by the color change reddish to yellow in the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube. The TSIA slant was used to detect the lactose fermenters and the saccharose and dextrose fermenters. The medium also helped to determine the ability of the organisms to produce H_2S. Pinkish slant and yellow butt or black slant and yellow butt were recorded as the positive reaction for *Salmonella*.

The MR test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. A red coloration was positive and indicated an acid pH of 4.5 or less resulting from the fermentation of glucose. A yellow coloration was considered as negative. In VP test, 2 milliliter of sterile glucose phosphate peptone water was inoculated with the 5 ml of test culture. It was observed closely for the slow development of a pink color for positive cases. Two milliliters of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours to conduct Indole test. Kovac's reagent (0.5 ml) was also added. A red color in the reagent layer indicated indole (Cheesbrough, 1985). The motility test was performed according to the method described by Cowan (1985) to differentiate motile bacteria from the non-motile one. The motile and non-motile organisms were identified by observing motility in contrasting with Brownian movement of bacteria. *Salmonella* agglutinating antiserum poly 'O' and poly 'H' of S & E reagents Lab, Bangkok, Thailand was used to do the serotyping of the isolated *Salmonella*. According to manufacturer's direction, it was noted that poly 'O' antiserum gives positive agglutination reaction with any serovars for preliminary screening of *Salmonella* and poly 'H' antiserum gives specific agglutination reaction for motile *Salmonella* (Santiviago *et al.*, 2001). The organisms isolated and identified as *Salmonella* were inoculated into the SSA slants and incubated at 37ºC for 24 hours in bacteriological incubatory. Then a loop full of thick bacterial culture was mixed with 20% sterile buffered glycerin in small vials and was preserved at 20ºC. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977). Susceptibility of the isolated *Salmonella* to different antibacterial agents as performed through disc diffusion method to determine the drug sensitivity pattern according to the method described by Bauser *et al.*, (1966). This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that result from different rates of diffusion of the agent into the medium surrounding the disc.

RESULTS AND DISCUSSION

Salmonella was detected in 16% of the collected samples under study (Table 1). NB inoculated separately with the collected samples revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and was indicated by the presence of turbidity. In the same way, the growth was indicated by the growth of circular, smooth, opaque, translucent colonies in nutrient agar plates streaked separately. On SSA plates, the organisms were produced pinhead or lentil sized, raised, round or circular smooth, glistening, opaque, colorless, transparent or translucent colonies and on BGA plates, they were produced pale pink color colonies against a pinkish background. In the case of MCA plates, the organisms were produced colorless, smooth colonies while they were produced black color colonies on TSIA slant. The thin smears prepared with the colony from SSA, MCA and BGA for Gram's staining revealed Gram-negative, pink colored, small rod shaped appearance, arranged in single or paired under the microscopic examination (Fig. 1). All the isolates were found to be motile having swinging movement when examined using hanging drop slide under microscope (Table 1).

Table 1. Detection of *Salmonella* serovars based on cultural, staining and morphological characters

Prevalence										
No. of sample	Media used	Change in broth	Cultural examination		Biochemical examination		Total positive sample (No.)			Detection (%)
			Positive to Salmonella	Negative to Salmonella	Positive to Salmonella	Negative to Salmonella	MMCH	BDC	Total	
25	NB, nutrient agar, MCA, SSA, BGA	Turbidity	4	21	4	21	3	1	4	16.00
Cultural, staining and morphological characteristics										
Sources of isolates	Colony characteristics						Staining characteristics			Motility
	SSA		MCA		BGA					
$MMCH_3$	Opaque, translucent, colorless, smooth and round colonies		Pale, colorless, smooth, transparent and raised colonies		Pale pink color colonies against a pinkish background		Gram negative and short rod shaped			+
$MMCH_8$	do		do		do		do			+
$MMCH_{14}$	do		do		do		do			+
BDC_5	do		do		do		do			+

All of the isolates fermented dextrose, maltose and mannitol with the production of acid and gas but did not ferment lactose and sucrose. Acid production was indicated by the color change from reddish to yellow and gas production was noted by the presence of gas bubbles in the inverted Durham's tubes (Fig. 2). All of the isolates were found to be indole negative, MR test positive and VP test negative (Fig. 3, Table 2). The rapid slide agglutination test with polyvalent 'O' (Poly 'O') and Polyvalent 'H' (poly 'H') antisera was conducted with all the isolated *Salmonella* serovars. In this test, all culturally and biochemically positive *Salmonella* serovars showed agglutination with poly 'O' and in case of polyvalent poly 'H', all isolates gave positive reaction (Fig. 4). In-vitro antibiotic sensitivity pattern of isolated Salmonellae was performed against 8 commonly used antibiotics belonging to different groups. After incubation, plates were examined and diameters of the zone of inhibition for individual antibacterial agents were designated as highly sensitive, moderately sensitive, less sensitive and resistant (Table 2).

Table 2. Characteristics of *Salmonella* serovars in biochemical and antibiotic sensitivity tests

Biochemical test								
Isolates	Carbohydrate fermentation test					Indole	MR	V-P
	Dextrose	Maltose	Lactose	Sucrose	Mannitol			
$MMCH_3$	+	+	-	-	+	-	+	-
$MMCH_8$	+	+	-	-	+	-	+	-
$MMCH_{14}$	+	+	-	-	+	-	+	-
BDC_5	+	+	-	-	+	-	+	-
Antibiotic sensitivity test								
Isolates	Antibiotic disc used							
	ER	AX	CP	CK	CT	KA	CI	NA
$MMCH_3$	+	+	+	+	++	++	+++	++
$MMCH_8$	-	+	+	++	+	++	+++	++
$MMCH_{14}$	-	+	++	++	+	+++	+++	++
BDC_5	+	+	+	+	++	+++	+++	++

Legends

ER	=	Erythromycin	CT	=	Cotrimoxazole	+++	=	Highly sensitive
AX	=	Amoxicillin	KA	=	Kanamycin	++	=	Moderately sensitive
CP	=	Cephalexin	CI	=	Ciprofloxacin	+	=	Less sensitive
CK	=	Chloramphenicol	NA	=	Nalidixic acid	-	=	Resistant

Among the isolates 100% were highly sensitive to ciprofloxacin, 80% and 60% were to kanamycin and chloramphenicol respectively while 40% to cotrimoxazole and 20% to cephalexin. However 40% were moderately sensitive to nalidixic acid and cotrimoxazole while 20% were kanamycin, chloramphenicol and cephalexin. 60%, 25% and 20% were found to be less sensitive to cephalexin, amoxicillin and cotrimoxazole Fig. 5, Fig. 6). As regard effectivity of the antibiotics, 100% were highly resistant to erythromycin and 75% resistant to amoxicillin while 20% were resistant to nalidixic acid and chloramphenicol (Table 3).

Table 3. Antibiotic sensitivity pattern

Sensitivity pattern	% of isolated strain sensitive to various antibiotics							
	ER	AX	CP	CK	CT	KA	CI	NA
Resistance	100	75	0	20	0	0	0	20
Less sensitive	0	25	60	0	20	0	0	0
Moderately sensitive	0	0	20	20	40	20	0	40
Highly sensitive	0	0	20	60	40	80	100	40

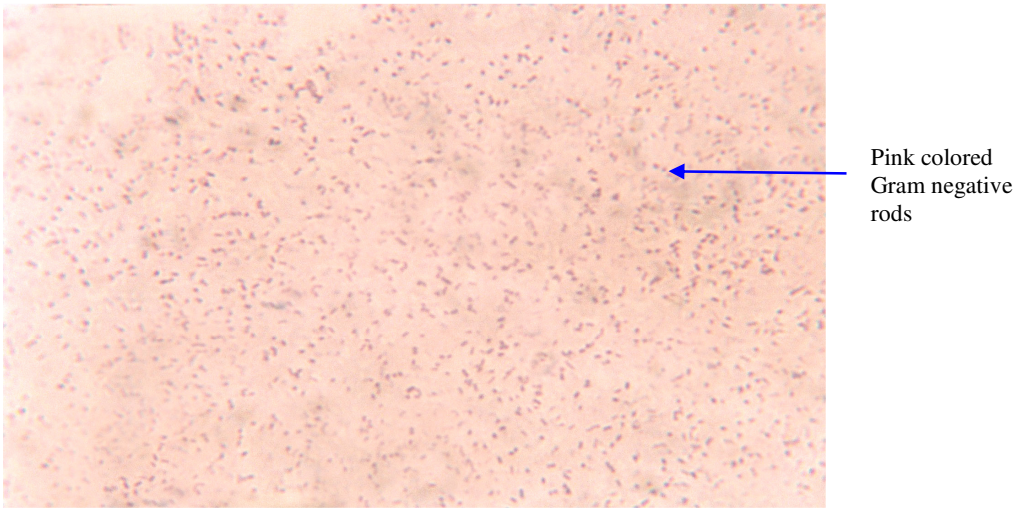

Fig 1. *Salmonella* serovars showing Gram negative small rods arranged singly or pairs on Gram's staining (100X)

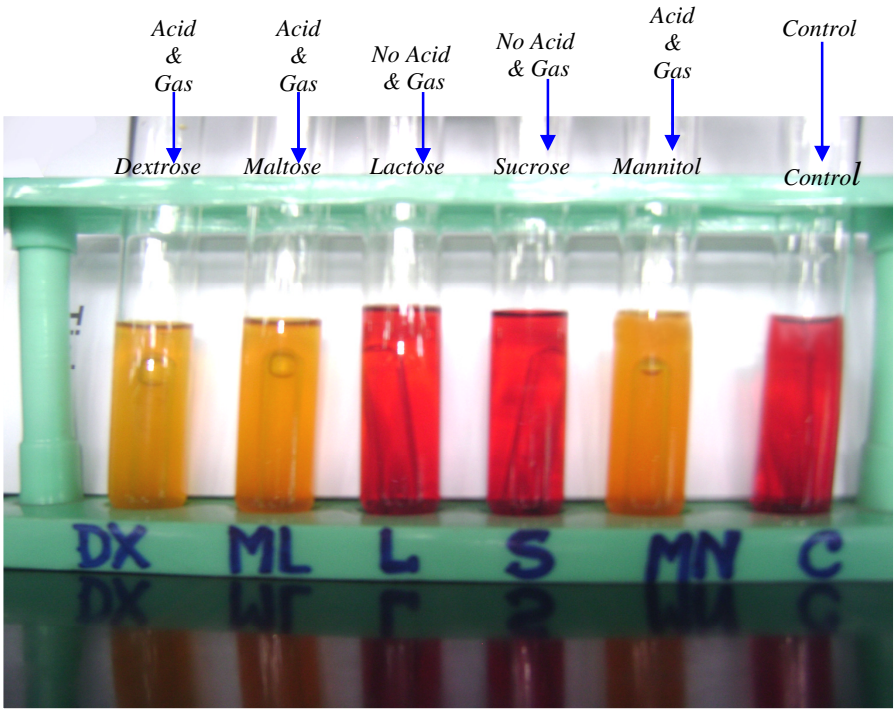

Fig 2. Sugar fermentation tests of *Salmonella*

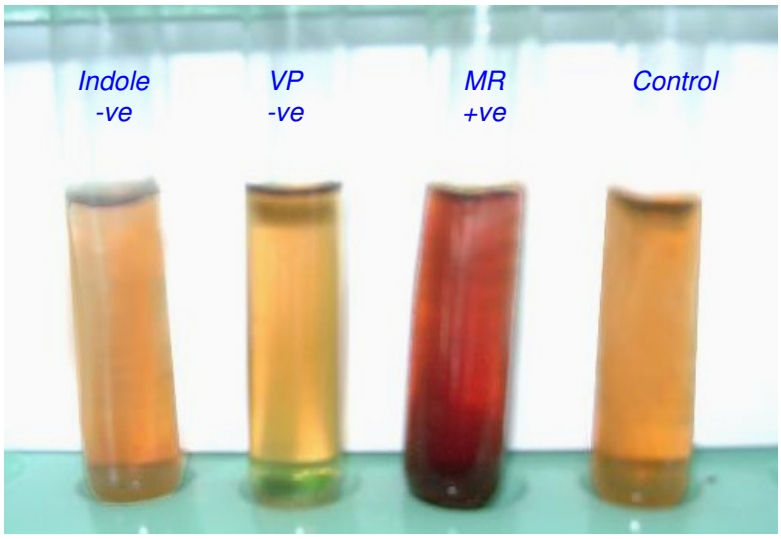

Fig 3. Indole, MR and VP tests

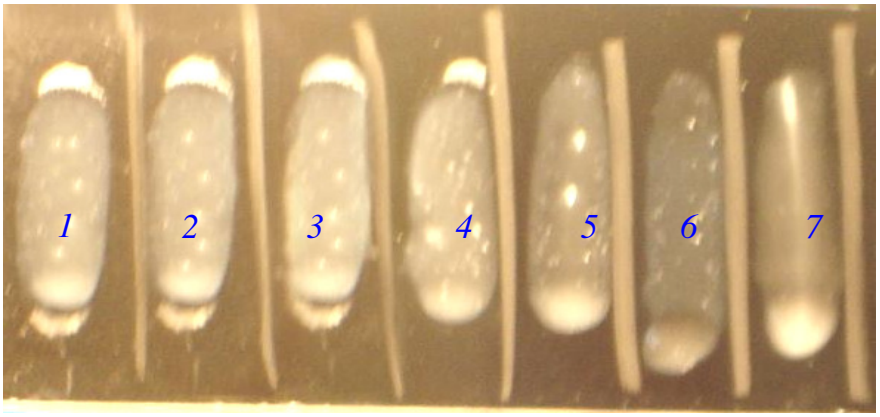

Fig 4. Serum plate agglutination test

1, 2, 3, 4, 5 = Agglutination, 6,7 = No Agglutination

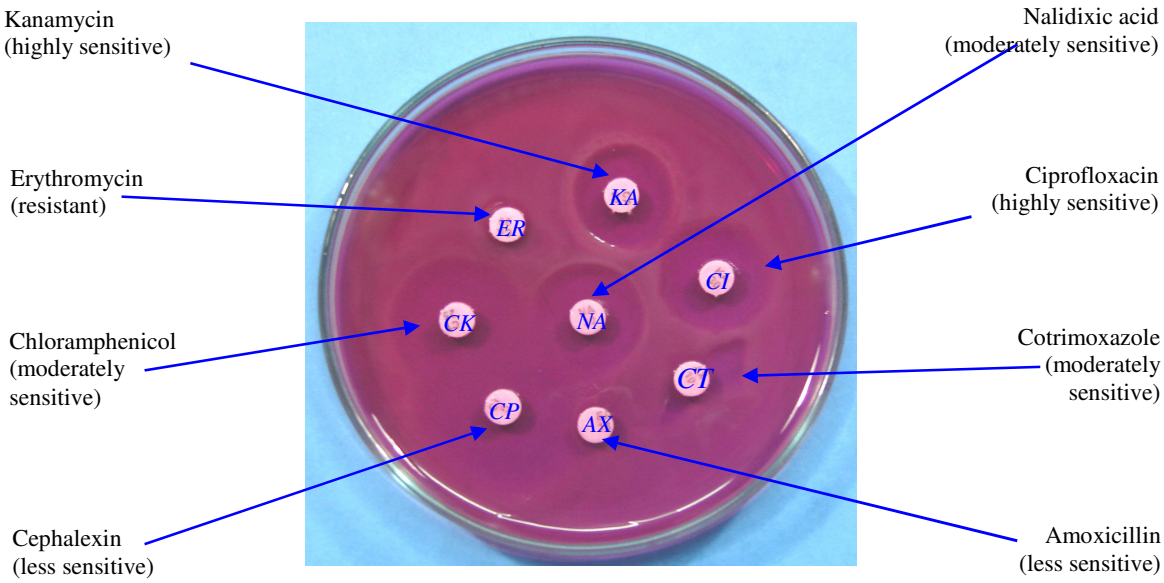

Fig 5. Antibiogram study of *Salmonella* serovars on BGA

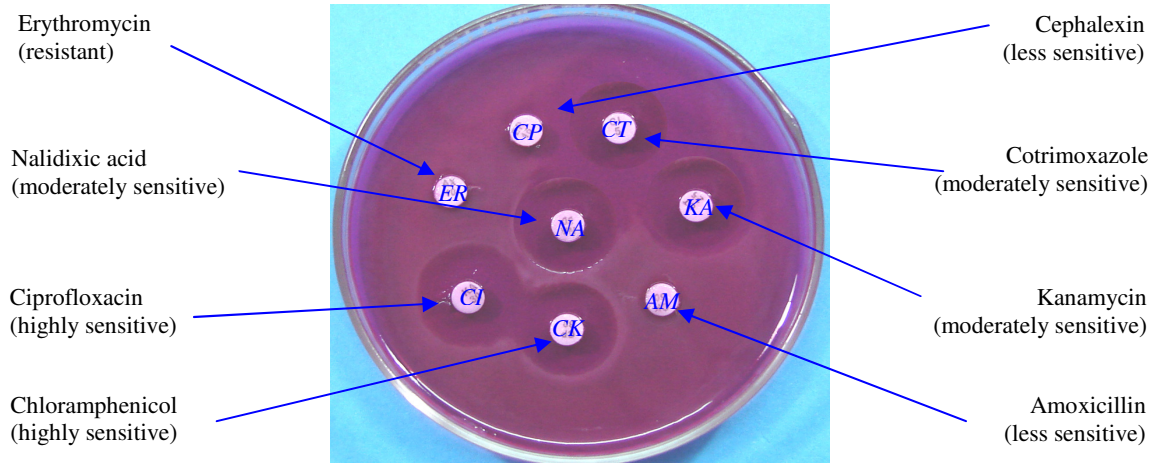

Fig 6. Antibiogram study of *Salmonella* serovars on BA

In Gram's staining, the morphological characteristics of the isolated *Salmonella* exhibited Gram negative, small rod shaped, single or paired in arrangement under microscope which was supported by Sogard *et al.* (2007) and Gene (2002). In motility test, all isolates were found to be motile and was correlated with the results of Buxton and Fraser (1977). *Salmonella* isolates were able to ferment the five basic sugars by producing both acid and gas. However differentiation of *Salmonella* into species level was difficult based on their sugar fermentation pattern. All the isolates of this study fermented dextrose, maltose and mannitol and produced acid and gas but did not ferment sucrose and lactose which satisfied the statement of Buxton and Fraser (1977), Hossain (2002) and Han *et al.* (2011). Moreover, all the isolates were found to be negative to indole tests positive to MR and negative to VP. In the present study, the prevalence rate of *Salmonella* is 16%. So, the results of this study are more or less in agree with the findings of the previous workers who also conducted research investigation on *Salmonella* from human stool sources. The slight differences among the prevalence percentages might be due to the species differentiation, hygienic, environmental and geographic variation and technical limitations of the laboratory of the study.

All culturally and biochemically positive *Salmonella* serovars showed agglutination with poly 'O' and in case of poly 'H' all showed positive reaction. From the antibiotic sensitivity study, it was exhibited that all isolates were highly sensitive to ciprofloxacin, kanamycin and chloramphenicol; moderately sensitive to cotrimazole and nalidixic acid; and less sensitive to cephalixin. Theses findings are in support of other studies like Adesiym *et al.*, (1988), Zhang *et al.*, (1998) and FDA (2010). The antibacterial resistance observed here in the isolated Salmonellae might be due to routine indiscriminate use of those antibacterial agents in field condition in study areas and/or rapid chromosomal mutation and presence of specific plasmid DNA. This provided the guideline to the veterinarians and physicians to select appropriate antibiotics to reduce economic loss through selecting the sensitive antibiotics.

The development and use of antibiotic has been one of the most important steps towards controlling of infectious bacterial diseases in 21st century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective (Kam *et al.*, 2007). To successfully fight the increasing number of drug resistant and multi drug resistance bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance and updated information is required. From the present study, it could be concluded that public health awareness should be developed to reduce the incidence of Salmonellosis among the people in order to avoid food borne illness. Proper treatment should be done with strict sanitary measures.

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