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Isolation and identification of Salmonella spp. from broiler and their antibiogram study in Sylhet, Bangladesh

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ABSTRACT

The objectives of this study were to isolate the associated *Salmonella spp.* from cloacal swabs of broiler and their antibiogram studies. A total of 80 cloacal swabs comprising of 50 samples of apparently healthy broiler and 30 samples of diarrheic broiler were collected from different poultry farms at Sylhet, Bangladesh during January to June 2013. The samples were subjected for isolation and identification of *Salmonella spp.* through a series of conventional bacteriological studies like study of morphology, staining properties, and biochemical characteristics. In results, 48% (n= 24/50) swab samples of healthy broiler and 66.7% (n= 20/30) diarrheic broiler were found to be associated with *Salmonella spp.* Among the 44 positive *Salmonella* isolates 47.73% (n=21) were *Salmonella pullorum*, 36.36% (n=16) isolates were *Salmonella gallinarum* and the rest 15.91% (n=7) isolates were *Salmonella typhimurium*. Sensitivity test was conducted against 10 commonly used antibiotics, of which Penicillin-G, Erythromycin, Ampicillin, and Bacitracin were found to be resistant, and Ceftriaxone, Gentamycin, and Choloramphenicol showed considerably better sensitivity as compared to others. It is concluded that *Salmonella spp.* are present in broiler, and the bacteria can be clinically controlled by using Ceftriaxone, Gentamycin, and Choloramphenicol.

1. INTRODUCTION

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector because of an almost assured in a relatively short period of time. Commercial poultry industry (mostly broiler and layer) plays an important role in the economy of Bangladesh. Little is known about the bacterial presence in the poultry environment such as in poultry litter and in the poultry house air [1]. The rural poultry production system in Bangladesh is typically a smallholder free range scavenging operation. Development of poultry sector in Bangladesh is being hampered by a number of factors, of which the diseases are considered as the major factor causing 30% mortality of chicken per year [2]. Intestinal bacteria play an important role on health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases and immune responses [3]. With the great expansion of poultry rearing and farming, Fowl typhoid caused by *S. gallinarum* is the most devastating disease in Bangladesh [4]. Important bacterial diseases of poultry in Bangladesh are Pullorum disease (PD) and Fowl Cholera (FC) which are responsible for high percentage of morbidity and mortality. Prevention of salmonella infection is important for the profitable expansion of poultry industry in Bangladesh. Salmonella infection is one of the most important bacterial diseases in poultry causing heavy economic loss through mortality and reduced production [5, 6]. Chicks can be infected with *Salmonella spp.* by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection and transportation of equipment and feed [7].

Age wise prevalence of avian Salmonellosis showed highest infection rate in adult layer (53.25%) in comparison to brooding (14.55%), growing (16.10%) and pullet (16.10%) [8]. Infections with the two non-motile serotypes *Salmonella pullorum* and *Salmonella gallinarum* which are generally host specific for avian species. Pullorum disease has great economic importance in poultry industry as it causes high mortality in young chicks, reduces growth rate in chicken and reduction of egg production in layer birds.

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Resistance against frequently used antibiotics has been observed in bacteria present in poultry since the introduction of these antimicrobial agents in poultry. The rise in antibiotic resistance has been reported in the past two decade in many countries including Bangladesh [9]. In Bangladesh, the economic aspect of poultry disease and their mortality and morbidity due to bacterial infection is a matter of great concern to the livestock owners. The antibiotic resistance pattern increases the incidence of disease in poultry and subsequently affects the economy of Bangladesh. Therefore, this study was designed to isolate and identify the *Salmonella spp.* from broiler and assessments of sensitivity of *Salmonella spp.* to different antibiotics.

2. MATERIALS AND METHODS

2.1 Study area

The sample (cloacal swab) were collected from apparently healthy and diarrheic broiler of different broiler farm located around SAU campus area (Baluchar, Alurtal, Shibgonj, Tilagore, Arambag, and Batassar) and then transported to the laboratory of the Department of Microbiology and Immunology, Sylhet Agricultural University Sylhet, Bangladesh during the period of January to June, 2013 for isolation, identification, and antimicrobial susceptibility testing.

2.2 Collection and transportation of samples

A total of 80 cloacal swab samples were collected and inoculated immediately into nutrient broth (NB) for better nourishment of the desirable organisms and immediately brought to Laboratory of the Department of Microbiology and Immunology, Sylhet Agricultural University Sylhet, Bangladesh.

2.6 Cultural characterization and isolation of *Salmonella spp.*

The samples were collected directly from cloaca by cotton buds and taken in nutrient broth and SSA (Salmonella-Shigella Agar) plates. These were then incubated at 37°C for 24 hours in bacteriological incubator. After 24 hours the incubated media were then examined for growth of bacteria. Colorless or translucent colony and sometimes black color colony were observed on SS agar. The colony was then subjected to Gram's Method of staining and observed under microscope for Gram negative rods. The organisms from the agar media were sub-cultured into SSA, MCA (MacConkey Agar), BGA (Brilliant Green Agar), EMBA (Eosin Methylin Blue Agar), and TSIA (Triple Super Iron Agar) with the help of inoculating loop in case of Gram negative rods in the smears. In case of SSA, colorless, translucent and black colony was observed. In case of MCA, colorless and translucent colony was observed. In case of BGA, light pink colony against a rose pink background was observed. In case of EMBA, pale colony without metallic sheen was observed. In case of TSIA, a black colored colony was observed. Thus single pure colony was obtained. These pure isolates obtaining in this way were used for the further study [11]. The Salmonellae colonies were characterized morphologically using Gram's stain according to the method described by [12]. The motility test was performed to differentiate motile bacteria from non-motile one [11].

2.7 Differentiation of isolated *Salmonella spp.* using biochemical test

For this study, isolated organisms with supporting growth characteristics of *Salmonella spp.* were subjected to sugar (Carbohydrate) fermentation test, TSIA slant reaction, MR (Methyl red) reaction, VP (Voges-Proskauer) reaction, indole reaction and citrate utilization reaction according to the procedures as described by [11].

2.8 Comparative antimicrobial sensitivity pattern of *Salmonella spp.*

Susceptibility and resistance of different antibiotics was measured in vitro by employing the Kirby-Bauer method [13, 14]. This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc. A suspension of test organism was prepared in NB (Nutrient Broth) by overnight culture for 24 hours at 37°C. The broth were streaked using sterile glass spreader homogenously on the medium. Antibiotic disc were applied aseptically on to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile pair of forceps on Mueller-Hinton agar plates. The plates were then inverted and incubated at 37°C for 24 hours. The diffusion discs with antimicrobial drugs were placed on the plates and incubated for 24 hours at 37°C. The antibiotics discs (Oxoid, Basingstoke, Hampshire, England) used were: Ampicillin, Penicillin-G, Nalidixic Acid, Erythromycin, Amoxycillin, Chloramphenicol, Gentamycin, Ceftriaxone, Bacitracin and Cefalexin. Sterile glass spreader was used to spread the culture homogenously on the medium. Antibiotic disc were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile pair of forceps. The plates were then inverted and incubated at 37°C for 24 hours. After incubation, the plates were examined and the diameters of the zone of complete inhibition were observed. Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation table (Table 6) updated according to the Clinical and Laboratory Standards Institution [15].

3. RESULTS AND DISCUSSION

About 80 cloacal swabs samples were collected for the research work. Cloacal swabs have been used to provide evidence of persistent intestinal colonization by salmonellae in individual birds [16]. Among 80 cloacal swabs samples 44 samples were positive. However *Salmonella spp.* was isolated from 24 out of 50 apparently healthy broiler samples and 20 out of 30 diarrheic broiler samples and the average positive isolates were 48% for healthy broiler samples and 66.67% for diarrheic broiler samples

(Table 1 and 2). The frequency distribution of *Salmonella spp.* isolates in different farm samples were found variable. However, each of the positive samples was treated as an isolate. Among the 80 samples tested from the apparently healthy and diarrheic birds in the broiler farms *Salmonella spp.* could be isolated from 44 (55%) samples which were in close agreement with [17]. The highest average infection rate (66.67%) was isolated from diarrheic broiler than the apparently healthy broiler (48%). So, the rate of isolation was higher than the findings of [18].

During the isolation, identification of bacterial colonies having typical cultural characteristics was selected as presumptive for *Salmonella* serovers. For this, general purpose and differential selective media such as SSA, BGA, TSIA and EMBA were used to culture the organism although all of them are not found equally suitable for all the serovars of *Salmonella* (Table 3). In the present study, specific enriched media and biochemical tests mentioned above were also used by a number of researchers [19, 20, 21, 22, 23]. The colony characteristics of *Salmonella spp.* such as translucent, black or colorless, smooth, small round colonies on SSA; black colored colonies on TSI agar were similar to the findings of other authors [19, 20, 21, 24, 25]. In Gram's staining, the morphology of the isolated *salmonellae* exhibited Gram negative characteristics of small rod shaped, single or paired in arrangement under microscope was reported by other researchers [11, 26]. Motility test was fundamental basis for the detection of motility or otherwise characteristics of *Salmonella* organisms [20, 21, 26]. In carbohydrate fermentation test, the isolates that fermented glucose, maltose and produced acid and gas but did not ferment lactose those indicated positive for *Salmonellae* as was stated by [20]. Among the 44 positive *Salmonella* isolates, 47.73% (where n=21) fermented glucose, maltose, rhamnose and produced both acid and gas but did not ferment dulcitol was considered positive for *S. pullorumum*. Only 36.36% isolates (where n=16) fermented glucose, maltose, dulcitol without producing acid and gas and did not ferment rhamnose indicating typical characteristics of *S. gallinarum*. The rest 15.91% isolates (n=7) fermented glucose, maltose, rhamnose and dulcitol with or without gas demonstrated provided indication of being *S. typhimurium* (Table 5). These observations are strongly correlated with the theme of [23, 24, 27]. A total of 44 (where n=44) isolates were positive for Methyl Red test but negative for VP test indicating characteristics of *Salmonella spp.* test which was similar with the statement of [25]. In indole test, all the test isolates (where n=44) did not develop any red color that indicated the *Salmonella* isolates were negative to indole test and this was similar with the findings of [23]. Organisms isolated from the collected samples under test, revealed unequivocal morphological, cultural and biochemical properties resembling *Salmonella spp.* as was recorded by [19, 20, 21, 26].

Among the 44 isolates (where n=44) of 21 isolates were similar to *Salmonella pullorum* and only 16 isolates showed the characteristics of *Salmonella gallinarum* and 7 isolates were more or less similar to *Salmonella typhimurium.* The study also indicated that the field sample contained Gram negative, rod shape and motile organism with various colony characteristics (large, smooth, round and sticky) in different bacteriological media. The isolates was able to produce characteristic black metallic sheen colonies on EMBA, pink colony on, pinkish colony on SSA.

Table 1: Isolation of *Salmonella spp.* from cloacal swab collected from broiler farms (apparently healthy broiler).

Sl. No.	Name of farms and Location	No. of samples collected	No. of samples positive for *Salmonella spp.*	Positive samples (%)
$F_1H_{10}B$	Baluchar Poultry Farm, Baluchar, Sonarbangla, Sylhet, Bangladesh	10	4	40
$F_2H_{14}B$	Lui Poultry Farm, Alurtal, Sylhet, Bangladesh	14	6	42.85
$F_3H_{16}B$	Delwar Broiler Farm, Mijortilla, Islampur, Sylhet, Bangladesh	16	9	56.25
$F_4H_{10}B$	Bhi-Bon Poultry farm, Bagmara, Sylhet, Bangladesh	10	5	50
		Total=50	Total=24	Av.=48%

Table 2: Isolation of *Salmonella spp.* from cloacal swab collected from broiler farms (diarrheic broiler).

Sl. No.	Name of farms and Location	No. of diarrheic birds	No. of sample collected	No. of samples positive for *Salmonella spp.*	Positive samples (%)
$F_1D_{10}B$	Al-manar Broiler Farm, Shaplabag, Tilagore, Sylhet, Bangladesh	85	10	8	80
F_2D_5B	3-star Poultry Farm, Gailapara, Batassar, Sylhet, Bangladesh	50	5	3	60
$F_3D_{10}B$	Chawdari Poultry Farm, Batassar, Sylhet, Bangladesh	107	10	7	70
F_4D_5B	Alurtal Poultry Farm, Alurtal, Sylhe, Bangladesh	70	5	2	40
			Total=30	Total=20	Av.=66.7%

FDB: Farms of diarrheic birds, Av.: Average.

Table 3: Result of Growth cultural and morphological characteristics of *Salmonella spp.*

Colony characteristics					Staining characteristics	Motility (Hanging drop method)
BGA	SSA	MCA	TSIA	EMBA		
Pale, colorless against a pinkish background	Opaque, translucent, colorless, smooth, round with black center.	Pale, colorless, smooth, transparent, raised colonies	Black colored colony	Pale colonies without Metallic sheen	Pink short rod, gram negative bacilli	+Ve(*S. typhimurium*) -Ve(*S. pullorum,S. gallinarum)*

Here, **BGA** = Brilliant Green Agar, **SSA** = Salmonella-Shigella Agar, **MCA** = MacConkey Agar, **TSIA** = Triple Super Iron Agar, **EMBA** = Eosin Methylin Blue Agar, **+** = Positive, **-** = Negative.

Table 4: Result of biochemical characteristics of *Salmonella spp.*

Indole Test	*MR* test	*VP* test	*Sugar fermentation test*					Citrate Utilization test
			Dextrose	*Sucrose*	*Lactose*	*Maltose*	*Mannitol*	
-	+	-	+	-	-	+	+	+

Here, MR = Methyl red, VP = Voges-Proskauer, + = positive reaction, - = negative reaction.

Table 5: Results of percentage (%) of *Salmonella* spp. available in cloacal swab.

Name of organism	Percentage (%) of *Salmonella spp.* available in cloacal swab	
	Cloacal swab (from apparently healthy broiler)	Cloacal swab (from diarrheic broiler)
Salmonella pullorum (21)	10 (46.62%)	11 (52.38%)
Salmonella gallinarum (16)	9 (56.25%)	7 (43.75%)
Salmonella typhimurium (7)	5 (71.43%)	2 (28.57%)
Total = 44	24	20

Table 6: Interpretive standards for disc diffusion susceptibility testing.

Name of Antibiotic disc	Disc concentration	Diameter of zone of Inhibition (mm)		
		Sensitive	Intermediate	Resistant
Ceftriaxone	30 µg	≥ 21	14-20	≤ 13
Gentamycin	10 µg	≥ 15	13-14	≤ 12
Choloramphanicol	30 µg	≥ 18	13-17	≤ 12
Cephalexin	30 µg	≥ 18	15-17	≤ 14
Amoxycillin	20 µg	≥ 18	14-17	≤ 13
Erythromycin	15 µg	≥ 23	14-22	≤ 13
Ampicillin	10 µg	≥ 17	14-16	≤ 13
Nalidixic acid	30 µg	≥ 19	14-18	≤ 13

µg = micro gram, mm = millimeter.

Table 7: Results of inhibitory zone in 6 samples with 10 antibiotics.

Antibacterial agents	Zone of inhibition (mm)						Average (mm)	Remarks	Choice of drug
	Sample number								
	1	2	3	4	5	6			
Ceftriaxone	35.8	36	36	36.2	36	35.6	35.93	Sensitive	**1st**
Gentamycin	28.8	30.5	27.5	35	29	32	30.46	Sensitive	2nd
Choloramphanicol	28.5	31	30	25.5	30	29.60	29.1	Sensitive	3rd
Cefalexin	21.5	17.5	18.3	20	19.1	20	19.4	Sensitive	5th
Amoxycillin	19	23.2	18	15.8	21.9	17.5	18.9	Sensitive	6th
Erythromycin	-	-	-	-	-	-	-	Resistant	-
Ampicillin	-	-	-	-	-	-	-	Resistant	-
Penicillin	-	-	-	-	-	-	-	Resistant	-
Nalidixic acid	18.7	21.5	17.5	20	17.7	19.5	22.15	Sensitive	4th
Bacitracin	-	-	-	-	-	-	-	Resistant	-

mm = millimeter.

The antibiotic sensitivity test against the Salmonellosis with commonly used 10 antibiotics such as Nalidixic Acid, Penicillin-G, Erythromycin, Ampicillin, Ceftriaxone, Gentamycin, Bacitracin, Cefalexin, Amoxycillin, and Chloramphenicol. The result of the inhibition of the bacterial growth was variable in different antibiotics (Table 7). Multidrug resistance was detected in case of apparently healthy bird's samples and diarrheic bird's samples. The results showed that out of 10 antibiotics, 4 antibiotics (Penicillin-G, Erythromycin, Ampicillin and Bacitracin) were resistant. *Salmonella* resistance to penicillin has also been reported by [28]. The present findings where *Salmonella spp.* resistant to erythromycin supported by [28, 29, 30, 31]. *Salmonella spp.* resistant to Ampicillin supported by [32]. Several strains of multiple antimicrobial resistant *Salmonella spp.* in chicken reported by [33]. Remaining 6 antibiotics were sensitive in which Ceftriaxone showed highest inhibitory zone about 35.93 mm in average of 6 samples. Gentamycin showed 2nd highest inhibitory zone about 30.46 mm in average of 6 samples. Choloramphanicol showed 3rd highest inhibitory zone about 29.1mm in average of 6 samples. These findings were more close to the previous result of [34]. The result of present findings where *Salmonella spp.* sensitive to Gentamycin supported by [29, 32, 35, 36, 37]. This result were similar to previous findings where *Salmonella spp.* sensitive to Chloramphenicol supported by [30, 31, 37, 38, 39]. The result of this test provided the guideline for the veterinarian to select appropriate antibiotics to reduce the economic loss through selecting the sensitive antibiotics for Salmonellosis which have public health significance.

4. CONCLUSION

Salmonella spp. were isolated and characterized successfully from broiler using different cultural, morphological examination, biochemical and antimicrobial susceptibility tests. The findings of the present study revealed the presence of multidrug resistant *Salmonella spp.* in broilers at Sylhet district of Bangladesh. There might be variation in strains of *Salmonella spp.* So, the genetic variation leading to highly resistant *Salmonella spp.* to constitute a threat for poultry industry in Bangladesh and also the findings of the experiment speculate that the use of

Ceftriaxone, Gentamycin, and choloramphanicol might have the preference in clinical control of Salmonellosis. Further studies calling for attention for future research might be molecular characterization and genomic studies to have an idea about genes responsible for pathogenecity and drug resistance of the isolates of Salmonellae from cloacal swab of broiler.

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