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Multidrug Resistance Pattern of *Salmonella* Typhimurium Isolated from Rectal Swabs of Stray Dogs at Chittagong Metropolitan Area (CMA), Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TMR, MSI, MNEA and MAH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMH, LA and AAF managed the analyses of the study. Author TD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A cross-sectional study was conducted to investigate the prevalence and antimicrobial resistance pattern in *Salmonella* from 108 rectal swab of stray dogs of 9 randomly selected areas of Chittagong Metropolitan Area. Rectal swabs were collected for isolating *Salmonella enterica* serovar Typhimurium through bacteriological culture and *invA* specific PCR assay followed by antimicrobial susceptibility testing. Out of the 108 samples, females showed higher prevalence (66.67%) than males (58.93%). Among the 67 bacterial culture positive isolates, 10.45% was *Salmonella* Typhimurium in InvA gene specific PCR. Isolated *Salmonella* was tested for resistance to twelve different antimicrobial agents, using disc diffusion method. In cultural sensitivity test, 100% resistance was found against Amoxicillin where higher resistance was found against Azithromycin, Cefixime, Ampicilin and Pefloxacin. Gentamycin and Colistin appeared to be sensitive. Multidrug resistance of *Salmonella* spp. has increased with a great deal in developing countries in the last decades. In this study, most of the *Salmonella* isolates were multidrug resistant. Rational use of antibiotics needs to be adopted in clinical practice to prevent the emergence of multi-drug resistance *Salmonella* and their zoonotic transmission.

Keywords: Salmonella typhimurium; antimicrobial resistance; MDR salmonella; stray dog.

1. INTRODUCTION

Antimicrobial resistance (AMR) amongst animals, particularly companion animals, is a complex area that is of increasing importance because of both patient factors and public health issues [1,2]. In recent years multidrug resistant (MDR) bacteria has taken the place of worry throughout the world. The pattern of resistance and transmission of resistance gene between bacteria is a matter of great concern. Indiscriminate use of antimicrobials is not uncommon in developing countries [1]. As a result, bacteria have become resistant in a rapid rate. Among MDR bacteria, *Salmonella* is one of the concerned bacteria throughout the world after several outbreaks in the developed world [3].

Salmonella, the etiological agent for both human and animal salmonellosis, can cause a very common and widely spread enteric disease. It is a significant cause of acute and chronic diarrhoea and death in numerous animal species and in human beings. Majority of *Salmonella* serovars can infect a wide host range [4]. However, faeces of nearly all animal species may be a potential source of *Salmonella*; therefore, the zoonotic transmission of *Salmonella* is not limited to food animals alone. Dogs that have close interaction with humans, may be responsible for *Salmonella* transmission. Veterinarians and public health officials have documented shedding Salmonellae by dogs as a possible source of *Salmonella* infection for dog owners and their communities [5,6].

There has been no previous study on antimicrobial resistance in faecal indicator bacteria from stray dogs in Bangladesh. There is paucity of information on the role of dogs as a potential source of *Salmonella* infection to humans despite an increase in dog-keeping among the elite living in the metropolitan cities. Considering the above facts present study was undertaken to assess the prevalence and multidrug resistance pattern of *Salmonella* spp. in faecal isolates recovered from rectal swab samples of stray dogs in Chittagong City Corporation, Bangladesh.

2. MATERIALS AND METHODS

2.1 Study Area

Chittagong Metropolitan area consists of 41 wards (administrative areas) having lots of stray and community dogs without registration and having continuous access to abattoir, human food processing units and also contact with children and adults. A total of 108 rectal swab samples were collected randomly from 9 locations of the study area during April to November, 2016.

2.2 Collection and Handling of Specimens

Dogs were restrained considering animal welfare and left immediately after sampling. Rectal swabs were collected using a sterile cotton swab and immediately transferred to Selenite Cysteine broth. Samples were shifted to clinical pathology

of Chittagong Veterinary and Animal Sciences University (CVASU) laboratory in a cool box containing ice.

2.3 Isolation and Identification of *Salmonella* Isolates

Selective enrichment was done in Selenite Cysteine broth (Oxoid, UK). Isolation of *Salmonella* was performed on Xylose Lysine Deoxycholate (XLD) medium (Oxoid, UK) and Triple Sugar Iron (TSI) (Oxoid, UK). Isolated bacteria were confirmed under the microscope by Gram's staining technique.

2.3.1 Presumptive isolation of *Salmonella* isolates

Primary selective enrichment was performed in Selenite Cysteine broth (Oxoid, UK). Specimens were incubated at 37℃ for overnight. Enriched isolates was streaked on Xylose Lysine Deoxycholate (XLD) medium (Oxoid, UK) and incubated for 24 hour at 37℃. Characteristic black centered colonies were suspected for the presence of *Salmonella*.

2.3.2 Purification of isolates

Isolated suspected colonies were transferred to Tryptone Soya Broth (TSB; Oxoid, UK) and incubated at 37℃ for 24 hours. Isolates were then preserved in 50% sterile glycerol at -20℃ with enriched TSB medium containing isolates.

2.3.3 Biochemical characterisation of *Salmonella* isolates

Presumptive isolates were streaked in Triple Sugar Iron (TSI) slant and incubated at 37℃ for 24 hours. Presence of *Salmonella* was indicated by black/yellow butt and red slant with gas production.

2.4 Cultural Sensitivity (CS) Test of *Salmonella* Isolates

After confirmation of isolates as *Salmonella* Typhimurium and *Salmonella* Entiritidis antimicrobial susceptibility of the isolates were determined by the micro disc diffusion method [7]. A number of 12 antibiotics were selected for CS test on the basis of their range of activity against enterobacteria on their use in dog by veterinarians and human medicine. Bacterial inoculums were prepared for CS test comparing with 0.5 McFarland standard. The inoculums were spread over the dried surface of Muller Hinton Agar (Oxoid, UK) following a rotation of 60 degrees of the swab stick in each time for three consequent passages with a view to getting equal spreading of inoculums. The antimicrobial disks were then placed on the surface of the streaked agar carefully keeping an adequate space among the discs. After dispensing all disks the agar plates were incubated at 37℃ for 18 hours followed by the measuring of the size of the zone of inhibition around a micro-disk with digital slide calipers and the result was deduced according to CLSI [7].

2.5 PCR Detection of *Salmonella* Typhimurium

Nucleic acid was extracted by boiling extraction method. Fresh culture colony from agar medium and 100 µl autoclaved phosphate buffer solution was vortexed and heated at 100℃ for 15 minutes and immediate freezing at -20℃ for 10 minutes. After centrifuging the sample at 13,000 rpm for 3 minutes extracted DNA product was collected from the supernatant and kept in eppendorf tube [8]. The presence of InvA gene was detected using a PCR protocol. Each PCR reaction consisted of 25 µLDreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 19 µL of nuclease-free water, 2 µL forward primer (5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′), 2 µL reverse primer (5′- TCA TCG CAC CGT CAA AGG AAC C-3′) and 2 µL DNA template. Running conditions were: 1 cycle of denaturation at 94°C for 60s, followed by 35 cycles of denaturation at 94°C for 60s, annealing at 56°C for 30s and elongation at 72°C for 30s, and a final cycle of elongation at 72°C for 7 min. The PCR products were electrophoresed using 1.2% agarose gel (Invitrogen Ultapure TM Agarose®-Carlsbad, USA) together with a 100bp DNA ladder at 130V followed by staining in Ethidium-bromide. The desired amplified DNA were visualised in UV illuminator at the level of 284bp band size [9].

3. RESULTS

3.1 Prevalence of *Salmonella* spp.

In traditional bacteriological culture technique, 67 (62.04%) specimens were found positive for *Salmonella* spp. The isolates from rectal swabs of nine (9) areas in Chittagong City Corporation, Bangladesh were evaluated for antimicrobial susceptibility to estimate the prevalence and

pattern of antimicrobial resistance and sensitivity among *Salmonella* spp. isolates.

3.2 Prevalence of *Salmonella* Typhimurium Using PCR Technique

Among the 67 isolates of *Salmonella* spp., 7 isolates (10.45%) were found positive for *Salmonella* Typhimurium by PCR (Fig. 3). That is the prevalence of *Salmonella* Typhimurium was (7/108) 6.49% in the study area.

3.3 Antimicrobial Resistance Pattern of *Salmonella* Isolates

The prevalence and pattern of antimicrobial resistance of *Salmonella* isolates has been outlined in Fig. 1. It shows the graphical presentation of resistance pattern of *Salmonella* isolates of stray dog. Resistance pattern of *Salmonella* isolates among the twelve tested antimicrobials, Amoxicillin and Ampicillin turned out as the highest level of resistance (100%) followed by Enrofloxacin (96.97%), Colistin (96.97%), Azithromycin (96.97%), Tetracycline (96.97%), Sulfonamide (93.94%), Pefloxacin (93.94%), Cefixime (93.94%), Erythromycin (78.79%), Gentamicin (66.67%), and Ceftriaxone (39.39%) in males while in female Amoxicillin and Azithromycin showed 100% resistance followed by Ampicillin (97.06%), Cefixime (97.06%), Oxytetracycline (97.06%), Pefloxacin (94.12%), Colistin (88.24%), Potentiated Sulfonamide (88.24%), Erythromycin (73.53%), Gentamicin (61.76%) and Ceftriaxone (47.06%). In both sexes of stray dogs, Ceftriaxone showed highest sensitivity (36.36&23.53%) against *Salmonella* isolates from different sampling sites and no isolates are found to be sensitive towards Amoxicillin, Ampicillin, Enrofloxacin, Cefixime and Azithromycin. Besides those antibiotics, Gentamicin showed 24.24% sensitivity followed by Erythromycin (6.06%), Pefloxacin (3.03%), Colistin (3.03%), Potentiated Sulfonamide (3.03%) and others (0%) in male and Gentamicin (20.59%), Colistin (11.76%), Potentiated Sulfonamide (5.88%), Oxytetracycline (2.94%), Erythromycin (2.94%) and others (0%). Patterns of multidrug resistance isolates of *Salmonella* in both sexes presented graphically in Fig. 2.

Among the twelve tested antimicrobials, resistance pattern of *Salmonella* isolates Amoxicillin turned out as the highest level of resistance ranged at (100%) followed by Azithromycin (91.67-100%), Cefixime (90-100%), Ampicillin (83.33-100%), Enrofloxacin (83.33-100%), Pefloxacin (83.33-100%), Potentiated Sulfonamide (66.67-100%), Tetracycline (50-100%), Colistin (50-100%), Gentamicin (0-100%), and Ceftriaxone (0-70%) across the study sites (Table 1).

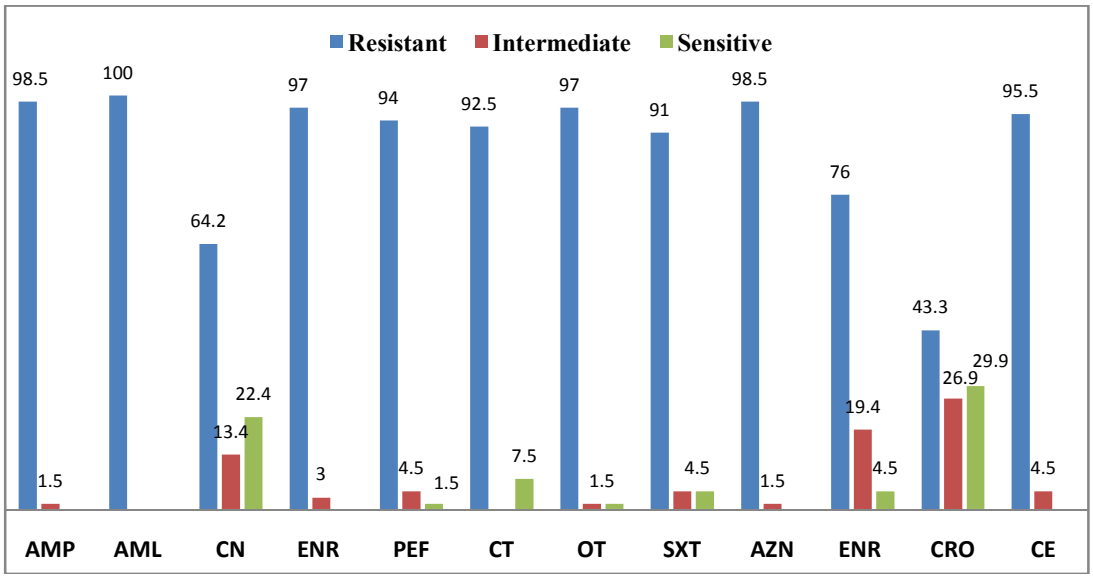

Fig. 1. Antimicrobial resistance pattern of *Salmonella* isolates

AMP: Ampicillin, AML: Amoxycillin, CN: Gentamicin, ENR: Enrofloxacin, PEF: Pefloxacin, CT: Colistin, OT: Oxytetracycline, SXT: Trimethoprim-Sulfamethoxazole, AZN: Azithromycin, ENR: Enrofloxacillin, CRO: Ceftriaxone, CE: Cefixime

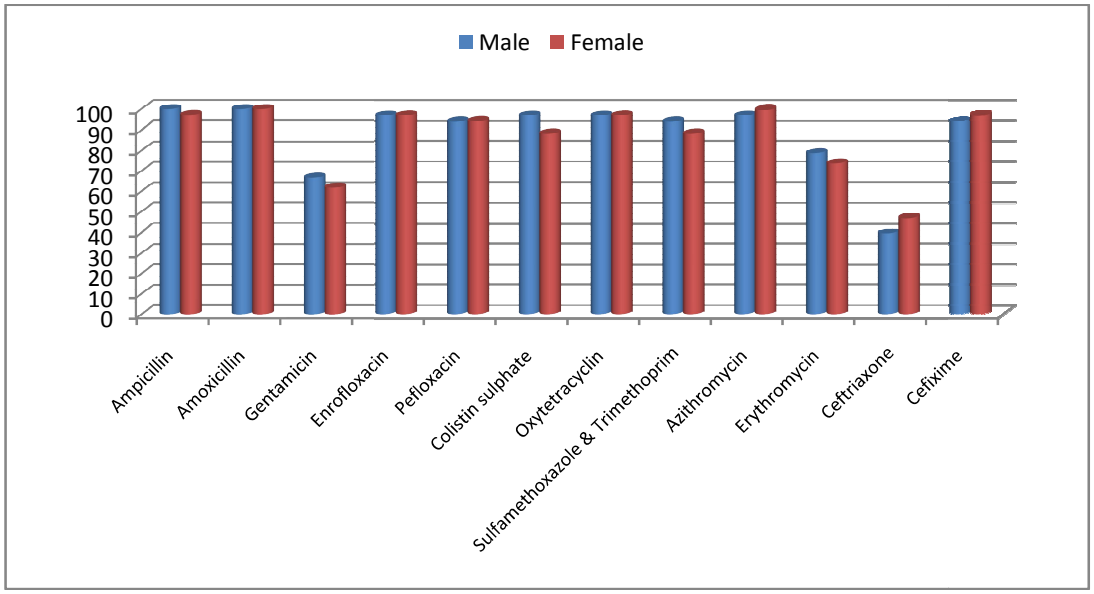

Fig. 2. Patterns of multidrug resistance isolates of *Salmonella* according to sex

AMP: Ampicillin, AML: Amoxycillin, CN: Gentamicin, ENR: Enrofloxacin, PEF: Pefloxacin, CT: Colistin, OT: Oxytetracycline, SXT: Trimethoprim-Sulfamethoxazole, AZN: Azithromycin, ENR: Enrofloxacillin, CRO: Ceftriaxone, CE: Cefixime

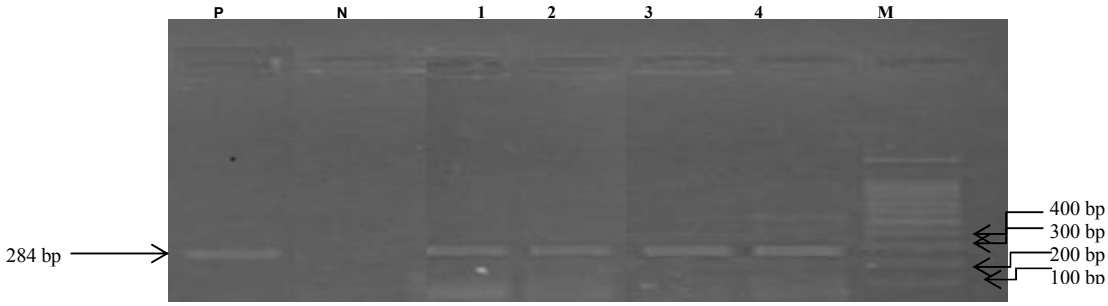

Fig. 3. PCR amplification of InvA gene of S. Typhimurium showing positive amplicons at 284bp

The PCR amplification of InvA gene of Salmonella showing positive amplicons at 284 bp on 1.5% agarose gel with ethidium bromide; M: DNA marker (100–1000 bp), Lane P: Positive control, Lane N: Negative control, Lane (1-4): Bacterial culture positive isolates

4. DISCUSSION

Stray dogs are common in Bangladesh and they feed on household wastage in dustbins, roadside restaurants, abattoir etc. and drink on sewage and drain water which is frequently contaminated with microorganisms. So they get infected frequently. On the other hand, stray dogs have a close attachment with people and children. Children often love to play with a dog nearby his house and also offer food. There is a high chance of cross infection to human. The aim of this study was to determine the occurrence of zoonotic *Salmonella* spp. in the faecal material along with estimating the prevalence of antimicrobial resistance against *Salmonella* spp. in randomly selected areas in Chittagong City Corporation of Bangladesh. To the best of our knowledge, this is the first study in Chittagong, Bangladesh to tackle this issue.

4.1 Prevalence of *Salmonella* spp. in Rectal Swab

Results of a retail surveillance conducted in Canada in 2003, showed S. Heidelberg to be the most prevalent serovar (73%) in 16% of chickens purchased from retail stores and markets, followed by S. Kentucky (11%). In Canada, S. Heidelberg was the most common cause of

human salmonellosis, accounting for 26% of all *Salmonella* isolates obtained from human cases through enhanced passive surveillance in 2003 [10]. *Salmonella* Typhimurium was the second most common serotype (25%) associated with human salmonellosis, followed by S. Enteritidis (15%) [11]. All the *Salmonella* isolates were identified as *Salmonella* Typhimurium in the study of Ojo and Adetosoye [12]. Seepersad singh, Adesiyun [13] reported 28 different serovars of *Salmonella*in dogs. *Salmonella* Typhimurium is the serotype most commonly isolated from dogs [14]. Human cases of Salmonellosis caused by *Salmonella* Typhimurium have been linked with likely contact with the faeces of infected dogs [5].

4.2 Prevalence of *Salmonella* Typhimurium in Rectal Swab

Salmonella Typhimurium is the serotype most commonly isolated from dogs [14]. Human cases of Salmonellosis caused by *Salmonella* Typhimurium have been linked with likely contact with the faeces of infected dogs (Anonymous). *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the most frequently isolated serovars from food borne outbreaks throughout the world [15,16]. Established conventional methods to detect and identify *Salmonella* are time consuming and include selective enrichment and plating followed by biochemical tests [17,18]. *In vitro* amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics [19,20]. Several genes have been used to detect *Salmonella* in natural environ-mental samples as well as food and fecal samples. Virulence chromosomal genes including; invA, invE, himAphoP are target genes for PCR amplification of *Salmonella* species [21]. *Salmonella* specific PCR with primers for invA is rapid, sensitive, and specific for detection of *Salmonella* in many clinical samples [22]. Ojo and Adetosoye [12] reported prevalence of *Salmonella* Typhimurium in non-diarrheic and diarrheic faeces were 4.0 and 3.7%, respectively which agree with present study (6.49%).

4.3 Antimicrobial Resistance Pattern of *Salmonella* Isolates

Approaches to prevent and control Salmonellosis in the food animal industry by various means such as improved biosecurity, vaccination, use of competitive exclusion products, and the introduction of novel immune-potentiators with limited success has necessitated the use of antimicrobial chemotherapy in the treatment and control of Salmonellosis [23,24]. The use of antimicrobials in food animals has resulted in the development of antimicrobial resistance [25,26], through mutation and acquisition of resistance encoding genes [27,28]. The situation in developing countries like Bangladesh may be exaggerated by easy accessibility of

Table 1. Pattern of multidrug resistance pattern of *Salmonella* isolated from rectal swab of dogs

Phenotypes of antibiotic resistance	Number of isolates
CN- ENR-AZN-OT-CRO-AMP-SXT-AML-PEF-CE-CT	11
CN-ENR-AZN-OT-CRO-AMP-SXT-AML-PEF-CE-E	1
ENR-AZN-OT-CRO-AMP-SXT-AML-PEF-CE-CT	1
CN-ENR-AZN-OT-AMP-SXT-AML-PEF-CE-CT	4
ENR-AZN-OT-AMP-SXT-AML-PEF-CE-CT	2
CN-ENR-OT-AMP-SXT-AML-PEF5-CE-CT	1
CN-ENR-OT-CRO-AMP-SXT-PEF-CE-CT-E	1
CN-ENR-AZN-OT-CRO-AMP-SXT-AML	3
ENR-AZN-OT-CRO-AMP-SXT-PEF-CE	1
ENR-AZN-OT-CRO-AMP-SXT-AML	5
CN-ENR-AZN-OT-AMP-SXT-AML	12
CN-ENR-AZN-OT-CRO-AMP-AML	1
ENR-OT-AMP-AML-PEF-CE-CT	1
ENR-AZN-OT-AMP-SXT-AML	8
ENR-AZN-OT-AMP-AML	1
ENR-AZN-OT-AML	1

AMP: Ampicillin, AML: Amoxycillin, CN: Gentamicin, ENR: Enrofloxacin, PEF: Pefloxacin, CT: Colistin, OT: Oxytetracycline, SXT: Trimethoprim-Sulfamethoxazole, AZN: Azithromycin, ENR: Enrofloxacillin, CRO: Ceftriaxone, CE: Cefixime

antimicrobials at a cheaper price and their extensive use in poultry production system [28,29]. Another major setback might be the quality and potency of locally produced antimicrobial drugs; for example, there are over 80 different brands of the Fluoroquinolones (Ciprofloxacin) in Bangladesh. Thus widespread availability and uncontrolled use of antibiotics poses the antimicrobial resistance in food animals and their products which might be the actual threat to public health. The current study recorded multiple antimicrobial resistances against *Salmonella* spp. (up to seven) and in most cases, estimated 100% resistance for category 2-4 antimicrobials across the study sites. These threats correspond to the many non-epidemiological and opportunistic earlier studies in Bangladesh [30,31], India [32], Nepal [33], Bhutan [34] and Malaysia [35].

Ojo and Adetosoye [12] stated 100% resistance against Erythromycin in *Salmonella* Typhimurium isolated from rectal swab of dogs in Nigeria. A similar result was found in several studies including Bangladesh [36,37]. Tetracycline showed higher resistance in the present study that are alarming for the layer farms and consumers of Bangladesh and the results agreed with the earlier researcher of Bangladesh [36,38] and India [39]. Earlier study in Bangladesh, Tetracycline reported 32% resistance from egg and its environment [30]. Contrarily 100% of *Salmonella* spp. cases were reported to be resistance to Tetracycline in India [40], 88% in Italy [41], Spain [42] and China [43,44]. In the present study we observed higher resistant of the *Salmonella* isolates against Enrofloxacin. The result was not agreed with Sing, (2012) where no isolate was found to be resistant against Enrofloxacin in north India. In Several investigations, resistance to Enrofloxacin were found to be 14% [45], 7% [46] in UK, 0.6% - 2% [47] in Australia that were comparatively lower than the current investigation. Pefloxacin is a Fluoroquinolones antimicrobial that is increasingly and successfully used for the treatment of Salmonellosis in humans and animals [47]. Among Fluoroquinolones, resistance to Pefloxacin was found comparatively lower in the present study as compared to 24% resistance in USA [48], 9 -14% in Germany [19]. Resistance to Ceftriaxone was recorded relatively lower proportions in study area which is consistent with the findings (0%) of Ojo and Adetosoye [12] in Nigeria. Resistances to Colistin among rectal isolates are reported from Senegal [49], Mexico [50] and USA [24]. Colistin resistance was comparatively increasing due to increasing use and recent availability in Bangladesh. The resistance pattern against Cefixime in our study was higher which is consistent with Ojo and Adetosoye [12] that reported 52.5% resistance from fecal isolates in dogs. Cefixime is not usually indicated in Salmonellosis in animals. We used this antibiotic to know the present response against *Salmonella*. There was found considerable intermediately sensitive isolates during this study. Gentamicin presented higher resistance in the present study in stray dogs. Ojo and Adetosoye [12] reported 35.3% resistance in fecal isolates of *Salmonella* spp. in dog which is consistent with our findings. In our study we found above 90% resistance against *Salmonella* spp. from rectal swab of stray dogs. This resistance might be due to frequent use of sulfonamides in animal and avian medicine in recent years in Bangladesh.

To know resistance pattern in identified *Salmonella* isolates, twelve commercially available antimicrobials were used in this study. *Salmonella* spp. was resistant to five of the twelve antimicrobials tested with simultaneous multidrug resistance to antimicrobials. A similar study in Turkey showed higher multidrug resistance in *Salmonella* spp. isolates as compared to humans [51]. *Salmonella* spp. was also found to be comparatively resistant to as many as 5 drugs tested in United States [52]. The increasing rates of resistance to Ampicillin, Amoxicillin, Tetracycline and Erythromycin among the isolates might be attributed to the emergence of multi resistance *Salmonella* spp. Multidrug resistance, with rates of resistance to Ampicillin, Chloramphenicol, and Trimethoprim-Sulfamethoxazole of more than 50%, has been reported in many areas of the world. Extended-spectrum Cephalosporins and Fluoroquinolones have been suggested as alternative agents in the treatment of infections caused by multidrug resistant *Salmonella* serotypes [47], these data correspond to results obtained in this study showing that the serotype isolated *S.* Typhimurium was resistant to considerable number of commercially used antimicrobials which carries a frightening information to policy makers and health care professionals. Resistance to antimicrobial agents in bacteria is mediated by several mechanisms including changes in bacterial cell wall permeability, energy dependent removal of antimicrobial agents via membrane-bound efflux pumps, modification of the site of drug action, and

destruction or inactivation of antimicrobial agents [47].

5. CONCLUSION

Salmonellosis is a leading food-borne and zoonotic disease worldwide. A wide range of foods and companion animals has been implicated in such disease. However, close living with an animal, especially pet animals and food animal products, have been consistently implicated in sporadic cases and outbreaks of human Salmonellosis. The results of the present study indicate that *Salmonella* contaminated dog faeces are common in the environment of Chittagong, Bangladesh. The poor sanitation and handling of sewage and slaughter house products in the city area could be a source of contamination. In the current study the prevalence of *Salmonella* spp. was higher in the rectal swab of stray dogs. *Salmonella* and antibiotic resistance was a big problem in Bangladesh. It is avowed that a larger number of resistant isolates of *Salmonella* for Ampicillin, Amoxicillin, Tetracycline, Enrofloxacin and Erythromycin. The excessive unregulated use of antibiotics in the dwells and veterinary practice, which might be the cause of increased resistance to Potentiated sulfur drugs, Pefloxacin and Colistin identified as sensitive drugs previously. The prevalence of MDR *Salmonella* Typhimurium in the rectal swabs of stray dogs indicates greater public health concern should be undertaken to prevent human salmonellosis.

ETHICAL APPROVAL

Ethical approval was taken from university ethical committee. The terms and conditions were maintained according to the guideline. No animal was harmed in this research.

DISCLAIMER

This paper is based on preliminary dataset. Readers are requested to consider this paper as preliminary research article. Authors are aware that detailed statistical analysis is required to get a scientifically established conclusion. Readers are requested to use the conclusion of this paper judiciously as statistical analysis is absent. Authors also recommend detailed statistical analysis for similar future studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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