Virulence and antimicrobial resistance profile of non-typhoidal *Salmonella enterica* serovars recovered from poultry processing environments at wet markets in Dhaka, Bangladesh

Running title: Virulence and antimicrobial resistance profile of NTS *enterica* serovars

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Abstract

The rapid emergence of virulent and multidrug-resistant (MDR) non-typhoidal *Salmonella* (NTS) *enterica* serovars are a growing public health concern globally. The present study focused on the assessment of the pathogenicity and antimicrobial resistance (AMR) profiling of NTS *enterica* serovars isolated from chicken processing environments at wet markets in Dhaka, Bangladesh. A total number of 870 samples consisting of carcass dressing water (CDW), chopping board swabs (CBS), and knife swabs (KS) were collected from 29 wet markets. The prevalence of *Salmonella* was found to be 20% in CDW, 19.31% in CBS and 17.58% in KS, respectively. Meanwhile, the MDR *Salmonella* was found to be 72.41%, 73.21% and 68.62% in CDW, CBS, and KS, respectively. All isolates were screened by polymerase chain reaction (PCR) for eight virulence genes, namely *invA*, *agfA*, *lpfA*, *hlyA*, *stxH*, *sefA*, *sopE*, and *spvC*. The *S. Enteritidis* and untyped *Salmonella* isolate harbored all virulence genes while *S. Typhimurium* isolates carried six virulence genes except *sefA* and *spvC*. Phenotypic resistance revealed decreased susceptibility to ciprofloxacin, streptomycin, ampicillin, tetracycline, gentamycin, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid and azithromycin. Genotypic resistance showed higher prevalence of plasmid mediated *bla*_{TEM} followed by *tetA*, *sul1*, *sul2*, *sul3*, and *strA/B* genes. Harmonic and symmetrical trend was observed among the phenotypic and genotypic resistance patterns of the isolates. The research findings anticipate that MDR and virulent NTS *enterica* serovars are prevailing in the wet market environments which can easily enter into the human food chain. There was a resilient and significant correlation existent among the phenotypic and genotypic resistance patterns and virulence genes of *Salmonella* isolate recovered from carcass dressing water, chopping board swabs, and knife swabs ($p < 0.05$), respectively.

Keywords: Antimicrobial resistance; Multidrug resistant; Non-typhoidal *Salmonella*; Virulence; Wet markets

Introduction

Salmonella has been recognized as one of the common pathogens that causes gastroenteritis (Hawker et al., 2019; Jain et al., 2020) with significant morbidity, mortality, and economic loss (Lin et al. 2014; Sallam et al., 2014). WHO reported 153 million cases of NTS enteric infections

worldwide in 2010, of which 56,969 were dead along with 50% were foodborne (Kirk et al., 2015). The disease surveillance report of China from 2006 to 2010 identified *Salmonella* as the second foodborne outbreak (Pang et al., 2011). NTS serovars like Typhimurium and Enteritidis are the predominant worldwide among the 2,600 serotypes of *Salmonella* have been identified (Issenhuth-Jeanjean et al., 2014; Takaya et al., 2019). Poultry has been regarded as the single prime cause of human salmonellosis and avian salmonellosis is not only affects the poultry industry but also can infect humans and caused by the consumption of contaminated poultry meat and eggs (Behraves et al., 2014). Poultry and eggs are considered to be the primary cause of salmonellosis and numerous other foodborne outbreaks (Gieraltowski et al., 2016; Keerthirathne et al., 2017; Biswas et al., 2019, 2020; Yu et al., 2020). Generally, *Salmonella* grows in animal farms may contaminate eggs and/or meat during the slaughtering process before being transferred to humans through the food chain. Indeed, numerous previous studies have been reported the isolation of *Salmonella* from foods of animal origin as well as human samples (Ed-Dra et al., 2018; Paudyal et al., 2020; Jiang et al., 2019; Elbediwi et al., 2020). Human *S. Enteritidis* are generally linked with the consumption of contaminated eggs and poultry meat, while *S. Typhimurium* with the consumption of pork, poultry, and beef (Park et al., 2014; Spector et al., 2012). Different prevalence of *Salmonella enterica* serovars has been reported around the globe from animal products and by-products (Park et al., 2014; De Freitas et al., 2010; Shah et al., 2012). *Salmonella* Typhimurium and Enteritidis are the most frequently reported serovars associated with human foodborne illnesses (Suresh et al., 2006). Untyped *Salmonella* of animal origin has been increasingly observed in Bangladesh (Momtaz et al., 2018; Sultana et al., 2014) but limited information has been published on *Salmonella enterica* serovars isolated from chicken processing environments and beyond.

Widespread uses of antimicrobials in poultry farming generate benefits for producers but aggravate the emergence of AMR bacteria (Manyi-Loh et al., 2018). Microorganisms that develop AMR are sometimes referred to as superbugs and open the door to treatment failure for even the most common pathogens, raise health care cost, and increases the severity and duration of infections. AMR burden may kill 300 million people during the next 35 years with a terrible impact on the global economy declining GDP by 2-3% in 2050 (O'Neill, 2014). WHO recognized AMR as a serious threat, is no longer a forecast for the future, which is happening around the world and affects everybody regardless of age, sex, and nation (Robicsek et al., 2006). Misuse and overuse of existing antimicrobials in humans, animals, and plants are accelerating the development and

spread of AMR (IACG, 2019). The antimicrobials are used in Bangladesh as the therapeutic, preventive and growth promoters in the poultry production system (Al Masud et al., 2020). The problem of AMR *Salmonella* emerged global concern in the modern decade (O'Bryan et al., 2018; Sultana et al., 2014). MDR *Salmonella* of poultry origin have been increasing in Bangladesh (Alam et al., 2020; Siddiky et al., 2021).

Usually, the virulence factors promote the pathogenicity of *Salmonella* infection. Chromosomal and plasmid-mediated virulence factors are associated with the pathogenicity of *Salmonella*. *Salmonella* possesses major virulence genes such as *invA*, *agfA*, *IpfA*, *hilA*, *sivH*, *sefA* and *sopE*. The infectivity of *Salmonella* strains is associated with various virulence genes existent in the chromosomal *Salmonella* pathogenicity islands (SPIs) (Nayak et al., 2004). The invasion genes *invA*, *hilA*, and *sivH* code with a protein in the inner chromosomal membrane of *Salmonella* that is necessary for the invasion to epithelial cells (Darwin et al., 1999). Moreover, *Salmonella* effector protein adhered by *sopE* gene which have relevance to *Salmonella* virulence (Huehn et al., 2010). The plasmid mediated *spvC* gene is responsible for vertical transmission (Silva et al., 2017). The long polar fimbria (*Ipf* operon) make the attraction of the microbes for Peyer's patches and adhesion to intestinal M cells (Bäumler et al., 1996). The aggregative fimbria (*agf* operon) promote the primary interaction of the *Salmonella* with the intestine of the host and stimulate microbial self-aggregation for higher rates of survival (Collinson et al., 1993). The *Salmonella*-encoded fimbria (*sef* operon) endorses interaction between the microbes and the macrophages (Collinson et al., 1993). Though there was a paucity of information in the determination of virulence gene from *Salmonella enterica* serovars in Bangladesh but recently eight virulence genes were found in *Salmonella* isolates of poultry origin in Bangladesh (Siddiky et al., 2021).

Wet markets are very common in Bangladesh which are commonly dirty, chaotic, and unhygienic and floors are constantly sprayed with water for washing and to conserve the humidity (Nidaullah et al., 2017). Dressing and processing of poultry in the open environment are common practices in the traditional wet markets. The chicken vendors himself dressing the chicken without having personal protective devices, without using clean dressing utensils such as chopping boards and knives. Even same water is used frequently for washing or cleaning the whole dressed carcass. There is a huge chance of cross-contamination and horizontal spreading of MDR *Salmonella* in the wet markets environment (Bupasha et al., 2020). The whole chicken carcass, vendor, and

consumer may be infected with *Salmonella* due to poor sanitary and hygienic practices. Even there is a great scope to spread and transmission of *Salmonella enterica* serovars in the agricultural food chain in the wet markets as most products are sold at ambient temperature and exposed to the environment (Sripaurya et al., 2019). A preceding study stated the incidence of *Salmonella* in various sites of the wet markets indicated a cause of cross-contamination in meat during selling through food contact surfaces or equipment (Nidaullah et al., 2017). Based on the importance of foodborne *Salmonella* at wet markets, this study was aimed to determine the pathogenicity and antimicrobial susceptibility profile of *Salmonella enterica* serovars isolated from poultry processing environments at wet markets in Dhaka, Bangladesh.

Materials and Methods

Study design and sample collection

The study was conducted in the 29 chicken wet markets around Dhaka city, the capital of Bangladesh from February to December 2019 in a cross-section manner (Fig 1). Dhaka city is called the biggest chicken selling hub due to the mass population density and economic sovereignty of the population. The sample size was calculated by using the “sample size calculator for prevalence studies, version 1.0.01” based on 25% prevalence of *Salmonella* spp. reported previously in Bangladesh (Naing et al., 2006; Daniel, 1999). The desired individual sample number should not less than 289. Three types of poultry processing environmental samples consisting of carcass dressing water (CDW), chopping board swabs (CBS) and knife swabs (KS) were collected independently as 290. The sterile cotton swabs contained in 10 ml buffered peptone water (BPW) were used for swabbing the samples. The samples were collected aseptically and immediately brought to the Antimicrobial Resistance Action Centre (ARAC) with an insulated icebox. This study received ethical approval from the Ethical Committee of the Animal Health Research Division at the Bangladesh Livestock Research Institute (BLRI), Dhaka, Bangladesh (ARAC: 15/10/2019:05).

Fig 1. Map of Dhaka city with location of 29 wet markets

Salmonella isolation and identification

Salmonella isolation and identification were carried out according to the guidelines of ISO (ISO, 6579:2002) as follows; pre-enrichment of the swab smear in BPW (Oxoid, UK) followed by aerobic incubation at 37°C for 18-24 h. Further, 0.1 mL of the pre-enriched sample was positioned discretely into three different locations on Modified Semisolid Rappaport Vassiliadis (MSRV; Oxoid, UK) agar and incubated at 41.5°C for 20-24 h. Then, a single loop from MSRV was streaked onto Xylose Lysine Deoxycholate (XLD; Oxoid, UK) and another loop onto MacConkey agar (Oxoid, UK) plates and incubated at 37°C for overnight. Typical black centered with reddish zone colony on XLD and colorless colony on MacConkey were picked up and afterward sub cultured on nutrient agar (NA; Oxoid, UK) and screened by biochemical tests: triple sugar iron (TSI), motility indole urea (MIU), catalase and oxidase. Final confirmation was done by the Vitek-2 compact analyzer (bioMérieux, France) followed by PCR (Siddiky et al., 2021).

DNA extraction

The DNA was extracted using conventional boiling method followed by a proven procedure (Sarker et al., 2019; Heidary et al., 2014). Briefly, the isolate was cultured on NA and incubated overnight at 37°C. Few fresh colonies were harvested from overnight culture and suspended in nuclease-free water. Then bacterial suspension was boiled at 99°C for 15 min followed by chilled on ice. Finally, the debris was separated by centrifugation and supernatant was taken as the DNA template for PCR.

PCR detection of *Salmonella* and *Salmonella enterica* serovars

Uniplex PCR (U-1) was performed to detect *Salmonella* species targeting virulence gene *invA* (Zahraei-Salehi et al., 2006). Multiplex PCR (M-I) was done to detect *S. Typhimurium* and *S. Enteritidis* (Agron et al., 2001; Alvarez et al., 2004). PCR reaction was adjusted in 25 µL mixture containing 2 µL of DNA template, 12.5 µL of 2x master mix (Go Taq Green Master Mix, Promega), 0.5 µL each of forward and reverse primers (10 pmol/µL) and 9.5 µL nuclease-free water. The PCR products were run at 100 V with 500 mA for 30 min in 1.5% agarose gel containing ethidium bromide. A 100bp DNA ladder (Thermo Scientific, USA) was used as a size marker. The primers used to detect *Salmonella* and *Salmonella enterica* serovars are presented in **Table 1**. *S.*

Typhimurium ATCC 14028 and *S. Enteritidis* ATCC 13076 were used as a positive control. Consequently, PCR positive *Salmonella* serovars Typhimurium and Enteritidis was further reconfirmed by the Vitek-2 compact analyzer (bioMérieux, France).

Antimicrobial Susceptibility Testing (AST)

AMR profile of all isolates was determined using the Kirby-Bauer disc diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2019). A panel of 16 antimicrobials representing 10 different classes were selected for AST consisting of aminoglycosides: amikacin (AK, 30µg), gentamicin (CN, 10µg), streptomycin (S, 10µg); carbapenem: meropenem (MEM, 10µg); cephalosporin/beta-lactam antibiotics: ceftriaxone (CRO, 30µg), cefotaxime (CT, 10µg), ceftazidime (CAZ, 30µg), aztreonam (ATM, 30µg); beta-lactamase inhibitors: amoxicillin-clavulanate (AMC, 30µg); penicillins: ampicillin (AMP, 10µg); macrolides: azithromycin (AZM, 15µg); quinolones/fluoroquinolones: ciprofloxacin (CIP, 5µg), nalidixic acid (NA, 30µg); folate pathway inhibitors: sulfamethoxazole-trimethoprim (SXT, 25µg); tetracycline: tetracycline (TE, 10µg); phenicols: chloramphenicol (C, 30µg). The isolates which were resistant to three or more classes of antibiotics were regarded as MDR (Magiorakos et al., 2012). The intermediate isolates were considered as resistant as the acquisition and transition from susceptible to resistance had already begun (Jaja et al., 2019). The positive control was used as *Escherichia coli* ATCC 25922. The multiple antibiotic resistance (MAR) index was calculated and interpreted using the proven method (Adzitey et al., 2012; Titilawo et al., 2015).

PCR detection of AMR genes

Phenotypically resistant *Salmonella* isolates were screened by PCR for the detection of 14 antibiotic resistance genes comprising of 7 β-lactamase genes (*bla*TEM, *bla*SHV, *bla*OXA, *bla*CTX-M-1, *bla*CTX-M-2, *bla*CTX-M-9 and *bla*CTX-Mg8/25), 3 tetracycline resistant genes (*tet*A, *tet*B and *tet*C), 3 sulfonamide resistant genes (*sul*1, *sul*2 and *sul*3) and single streptomycin resistant gene (*str*A/B). For β-lactam gene, two cycles of multiplex PCR (M-II & M-III) were carried out following the proven method of Dallenne et al. (2010). Consecutively, two cycles of multiplex PCR (M-IV & M-V) were performed to detect the resistance genes for sulfonamide, tetracycline and streptomycin in consistent with established method (Kozak et al., 2009). PCR reaction mixture, as well as gel electrophoresis was done in alignment with the procedures applied

for the detection of *Salmonella enterica* serovars in this study. The primers used to detect resistance genes are presented in **Table 1**.

Table 1. Primers used to detect *Salmonella enterica* serovars and resistance genes

PCR	Sequence	Amplicon size (bp)	Thermal Profile	References
U-I	F-GTGAAATTATCGCCACGTTCCGGGCAA R-TCATCGCACCGTCAAAGGAACC	284	95°C for 1 min; 38 cycles of 95°C for 30s, 64°C for 30s and 72°C for 30s; elongation step at 72°C for 4 min	Malorny et al., 2003
M-I	F-TTGTTCACTTTTACCCCTGAA R-CCCTGACAGCCG TTAGATATT	401	95°C for 2 min; 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 2 min; elongation at 72°C for 5 min	Alvarez et al., 2004
	F-TGTGTTTATCTGATGCAAGAGG R-CGTTCTTCTGGTACTTACGATGAC	293		Agron et al., 2001
	F-CCGTTTCATCCATAGTTGCCTGAC R-TTTCCTGTGCGCCCTTATTC	800		Dallenne et al., 2010
M-II	F-AGCCGCTTGAGCAAATTAAAC R-ATCCCGCAGATAAATCACCAC	713	94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; elongation step at 72°C for 7 min	Dallenne et al., 2010
	F-GGCACCAGATTCAACTTCAAG R-GACCCCAAGTTTCTGTAAGTG	564		Dallenne et al., 2010
	F-TTAGGAARTGTGCCGCTGYA R-CGATATCGTTGGTGGTRCCAT	688		Dallenne et al., 2010
M-III	F-CGTTAACGGCACGATGAC R-CGATATCGTTGGTGGTRCCAT	404	94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; elongation step at 72°C for 7 min	Dallenne et al., 2010
	F-TCAAGCCTGCCGATCTGGT R-TGATTCTCGCCGCTGAAG	561		Dallenne et al., 2010
	F-AACRCRCAGACGCTCTAC R-TCGAGCCGGAASGTGTYAT	326		Dallenne et al., 2010
	F-CGG CGT GGG CTA CCT GAA CG R-GCC GAT CGC GTG AAG TTC CG	433		Kozak et al., 2009
M-IV	F-CGG CGT GGG CTA CCT GAA CG R-GCC GAT CGC GTG AAG TTC CG	721	95°C for 15 min; 30 cycles of 95°C for 1 min, 66°C for 1 min and 72°C for 1 min; elongation step at 72°C for 10 min	Kozak et al., 2009
	F-CAACGGAAGTGG GCGTTG TGGA R-GCT GCA CCA ATT CGC TGAACG	244		Kozak et al., 2009
	F-GGC GGTCTT CTT CAT CATGC R-CGG CAG GCA GAG CAA GTAGA	502		Kozak et al., 2009
M-V	F-CGC CCA GTG CTG TTG TTGTC R-CGC GTT GAG AAG CTG AGG TG	173	94°C for 15 min; 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min; elongation step at 72°C for 10 min	Kozak et al., 2009
	F-GCT GTAGGCATAGGCTTGGT R-GCC GGA AGC GAG AAGAATCA	888		Kozak et al., 2009
	F-ATGGTGGACCCTAAACTCT R-CGTCTAGGATCGAGACAAAG	893		Kozak et al., 2009

PCR detection of virulence genes in *Salmonella* isolates

All *Salmonella* isolates were screened for the determination of eight important virulent genes encoding *invA*, *agfA*, *IpfA*, *hilA*, *sivH*, *sefA*, *sopE* and *spvC*. The PCR was executed in single reactions following previously used specific primers and thermal profiles (Zahraei-Salehi et al., 2006; Cesco et al 2008; Bäumlér & Heffron 1995; Guo et al., 2000; Kingsley et al., 2003; Oliveira et al., 2002; Prager et al., 2003; Swamy et al., 1996). PCR reaction mixture, as well as gel electrophoresis was done in alignment with the procedures applied for the detection of *Salmonella enterica* serovars in this study. The reference positive control (*S. Typhimurium* ATCC 14028 and *S. Enteritidis* ATCC 13076) and negative control (*E. coli* ATCC 25922) were used for validation. The primers used in this study are presented in **Table 2**.

Table 2. Primers used to detect virulence gene in *Salmonella* isolates

PCR	Target Gene	Sequence	Amplicon size (bp)	Thermal Profile	References
P-1	<i>agfA</i>	F-TCCACAATGGGGCGGCGGCG R-CCTGACGCACCATTACGCTG	350	94°C for 1 sec; 58°C for 1 sec; 74°C 21 sec	Cesco et al 2008
P-2	<i>IpfA</i>	F-CTTTCGCTGCTGAATCTGGT R-CAGTGTTAAACAGAAACCAGT	250	94°C for 1 sec; 55°C for 1 sec; 74°C 21 sec	Bäumlér & Heffron 1995
P-3	<i>hilA</i>	F-CTGCCGCACTGTTAAGGATA R-CTGTCGCCTTAATCGCATGT	497	94°C for 120 sec; 62°C for 1 min; 72°C 1 min	(Guo et al., 2000)
P-4	<i>sivH</i>	F-GTATGCGAACAAGCGTAACAC R-CAGAATGCGAATCCTTCGCAC	763	94°C for 30 sec; 56°C for 45 sec; 72°C 45 sec	(Kingsley et al., 2003)
P-5	<i>sefA</i>	F-GATACTGCTGAACGTAGAAGG R-GCGTAAATCAGCATCTGCAGTAGC	488	94°C for 1 sec; 56°C for 1 sec; 74°C 21 sec	(Oliveira et al., 2002)
P-6	<i>sopE</i>	F-GGATGCCTTCTGATGTTGACTGG R-ACACACTTTCACCGAGGAAGCG	398	94°C for 1 min; 55°C for 1 min; 72°C for 1 min	(Prager et al., 2003)
P-7	<i>spvC</i>	F-CCCAAACCCATACTTACTCTG R-CGGAAATACCATCTACAAATA	669	93°C for 1 min; 42°C for 1 min; 72°C for 2 min	(Swamy et al., 1996)

Statistical analysis

The antimicrobial susceptibility data were presented in Excel sheets (MS-2016) and analyzed by SPSS software (SPSS-24.0). The prevalence was calculated using descriptive analysis and the Chi-square test was applied to determine the level of significance. The statistical significance was determined by *p*-values less than 0.05 ($p < 0.05$).

Results

Prevalence of NTS *enterica* serovars

Of all 870 samples, 165 (18.96%) were found to be positive for *Salmonella*. The prevalence of *Salmonella* was found 20% (58 in 290) in CDW, 19.31% (56 in 290) in CBS, and 17.58% (51 in 290) in KS ($p < 0.05$). Meanwhile, the MDR *Salmonella* was found to be 72.41% (42 in 58), 73.21% (41 in 56) and 68.62% (35 in 51) in CDW, CBS, and KS, respectively ($p < 0.05$). The overall prevalence of *S. Typhimurium*, *S. Enteritidis*, and untyped *Salmonella* was found to be 8.96%, 1.6%, and 8.38%, respectively along with an overall MDR were 71.41%. The prevalence of NTS Typhimurium, Enteritidis and untyped *Salmonella* were found to be 7.93%, (23 in 290) 1.72% (5 in 290) and 10.34% (30/290) in CDW, respectively. Likewise, the prevalence of NTS Typhimurium, Enteritidis, and untyped *Salmonella* was found to be 10.34% (30 in 290), 2.06% (6 in 290) and 6.89% (20/290) in CBS, respectively. Consecutively, the prevalence of NTS Typhimurium, Enteritidis, and untyped *Salmonella* were represented 8.62% (25 in 290), 1.03% (3 in 290) and 7.93% (23 in 290) in KS, correspondingly. The prevalence of NTS Typhimurium in CBS were significantly higher compared to CDW and KS ($p < 0.05$).

Phenotypic resistance patterns of NTS isolates

AST in CDW revealed the highest resistance to ciprofloxacin (68.95%) followed by nalidixic acid (62.06%), tetracycline (60.33%), ampicillin, (58.61%) and streptomycin (56.88%); moderate resistance to gentamicin (39.64%), amoxicillin-clavulanate (31.92%), sulfamethoxazole-trimethoprim, (27.58%) and chloramphenicol (20.67%). On the contrary, low resistance was observed to azithromycin, amikacin and meropenem, respectively. Third-generation cephalosporins (ceftriaxone, cefotaxime, ceftazidime, and aztreonam) were found almost sensitive to all *Salmonella* isolates recovered from CDW (**Fig 2A**). Consecutively, AST of CBS showed higher resistance to streptomycin (64.28%) followed by ciprofloxacin (62.49%), ampicillin (62.27%), tetracycline (60.7%), nalidixic acid (53.56%), and gentamicin (53.56%); moderate resistance (14.27%-46.41%) were recorded to sulfamethoxazole-trimethoprim, amoxicillin-clavulanate, chloramphenicol and azithromycin. Besides, complete sensitive were found in all third-generation cephalosporins including carbapenem (ceftriaxone, cefotaxime, ceftazidime, aztreonam, and meropenem) (**Fig 2B**). Successively, AST of KS exhibited higher resistance to ciprofloxacin (64.69%), ampicillin (64.69%), streptomycin (64.7%), nalidixic acid (58.81%), and

tetracycline (54.89%); moderate resistance was recorded to gentamicin (47.05%), sulfamethoxazole-trimethoprim (47.05%), amoxicillin-clavulanate (27.44%) and chloramphenicol (21.56%). On the contrary, very low resistance or almost sensitive were observed to azithromycin, amikacin, meropenem, and third-generation cephalosporins (ceftriaxone, cefotaxime, ceftazidime, and aztreonam) (**Fig 2C**). There was harmony and synergy among the phenotypic resistance patterns of CDW, CBS, and KS. The AST pattern of ciprofloxacin in CDW was significantly higher compared to CBS ($p < 0.05$). Similarly, the AST pattern of gentamicin in CBS were significantly higher compared to CDW and KS ($p < 0.05$). The highest MAR index value of 0.68, 0.62, and 0.56 was recorded in KS, CDW and CBS, respectively ($p < 0.05$). The complete sensitive isolates were identified at 1.39% (4 in 290), 2.06% (6 in 290) and 1.03% (3 in 290) in the CDW, CBS, and KS, respectively. The most common antibiotic resistance profile CIP-S-AMP-TE-NA-CN was observed in the *Salmonella* isolates. The detailed phenotypic susceptibility pattern, including *Salmonella enterica* serovars and untyped *Salmonella* from three different sources, is presented in graphical form (**Figs 2A-C**).

Fig 2. AMR patterns of carcass dressing water (A), chopping board swabs (B) and knife swabs (C).

Genotypic resistance patterns of NTS isolates

All phenotypically resistant *Salmonella* isolates were screened by PCR for the detection of 14 antibiotic resistant genes encompassing of 7 β -lactamase genes (*bla*TEM, *bla*SHV, *bla*OXA, *bla*CTX-M-1, *bla*CTX-M-2, *bla*CTX-M-9 and *bla*CTX-Mg8/25), 3 tetracycline resistant genes (*tet*A, *tet*B and *tet*C), 3 sulfonamide resistant genes (*sul*1, *sul*2, and *sul*3) and single streptomycin resistant gene (*str*A/B) recovered from CDW (**Fig 3A**), CBS (**Fig 3B**) and KS (**Fig 3C**).

Out of seven, only one ESBL gene, *bla*TEM was detected with a prevalence rate of 62.06%, 69.62% and 62.73% in CDW, CBS, and KS, respectively. Consecutively, out of three tetracycline resistant genes, only one *tet*A was identified with the prevalence level of 60.32%, 58.92%, and 58.81% in CDW, CBS, and KS, respectively. Sequentially, the prevalence of *sul*1 gene were found 60.33%, 69.62%, and 49.01% in CDW, CBS, and KS, respectively. Furthermore, *sul*2 and *sul*3 were also found only in CBS with lower prevalence level of 3.56% and 3.56%, respectively. Similarly, *sul*3 gene was detected only in KS with a prevalence rate of 17.62%. Moreover, the streptomycin resistance gene, *str*A/B was detected with a prevalence rate of 36.2%, 24.99%, and

31.36% in CDW, CBS and KS, respectively. The detailed genotypic susceptibility pattern, including *Salmonella enterica* serovars and untyped *Salmonella* from three different sources, is presented in graphical form (Figs 3A-C). The *sul1* gene in CBS were significantly higher compared to CDW and KS ($p < 0.05$). Similarly, *strA/B* gene in CDW was significantly higher compared to KS and CBS ($p < 0.05$). There was a strong correlation and synergy persists among the genotypic features.

Fig 3. Genotypic resistance patterns of carcass dressing water (A), chopping board swabs (B) and knife swabs (C).

PCR detection of virulence genes for NTS isolates

All *Salmonella* isolates were screened by PCR to monitor eight common virulence genes namely *invA*, *agfA*, *IpfA*, *hilA*, *sivH*, *sefA*, *sopE*, and *spvC*. *S. Enteritidis* and untyped *Salmonella* isolates were found positive for all eight common virulence genes whereas *S. Typhimurium* harbored six virulence genes (Fig 4). There was a strong correlation exist among the virulence genes in CDW, CBS, and KS ($p < 0.05$).

Fig 4. Virulence genes distribution among *Salmonella* isolates

Discussion

Very few studies have reported NTS *enterica* serovars isolated from poultry processing environments at wet markets in Bangladesh. In our study, we found the overall prevalence of *Salmonella* 18.96% in poultry processing environmental samples including CDW, CBS and KS. Previously, the prevalence of *Salmonella* in Bangladesh was found 23.33% in poultry slaughter specimens (Momtaz et al., 2018); 26.6% in chicken cloacal swab, intestinal fluid, egg surface, hand wash, and soil of chicken market samples (Akond et al., 2012); 25.35% in the chicken cloacal swab, eggshells, intestinal contents, liver swabs, broiler meat, and swabs of slaughterhouse (Karim et al., 2017); 35% in broiler farms settings (Alam et al., 2020); 23.53% in poultry samples (Al Mamun et al., 2017); 37.9% in poultry production settings (Mahmud et al., 2011); 31.25% in broiler farm settings (Mridha et al., 2020); 42% in broiler chicken (Sarker et al., 2021) and 65% in frozen chicken meat (Parvin et al., 2020). Successively, Siddiky et al. (2021) found the prevalence of *Salmonella* 8.62% in broiler, 6.89% in sonali and 3.1% in native chicken cecal contents. The

prevalence of *Salmonella* isolates in our findings was compatible with previous data in Bangladesh.

Continually, the overall prevalence of *Salmonella* was found at 17.3% in butcher shops in Ethiopia. They identified *Salmonella* in KS, CBS, hand washings, and meat which has strong synergy with our findings (Garedew et al., 2015). Rather, the wet market based study in India revealed the prevalence of *Salmonella* 14.83% in chicken meat shops (Sharma et al., 2019); 19.04% in retail chicken shops (Waghamare et al., 2017), and 23.7% in white and red meat at local markets (Kaushik et al., 2014). A study demonstrated the high prevalence of *Salmonella* (88.46%) in poultry processing and environmental samples obtained from wet markets and small-scale processing plants in Malaysia (Nidaullah et al., 2017). Rusul et al. (1996) reported the prevalence of *Salmonella* 35.5% and 50.0% in broiler carcasses at the wet markets and processing plants, respectively. Furthermore, the overall prevalence of *Salmonella* serovars identified 23.5% in ducks, duck rearing, and processing environments in Penang, Malaysia (Adzitey et al., 2012). Moreover, the recent prevalence of *Salmonella* at home and abroad was linked to our findings. In our study, the prevalence of NTS Typhimurium was found to be 7.93%, 10.34%, and 8.62% respectively in CDW, CBS and KS. Consecutively, the occurrence of *S. Enteritidis* were found 1.72%, 2.06%, and 1.03% in CDW, CBS, and KS respectively. Siddiky et al. (2021) found the overall prevalence of *S. Typhimurium* and *S. Enteritidis* at the rate of 3.67% and 0.57% in chicken cecal contents. Some correlation of the prevalence of *Salmonella enterica* serovars between caecal content and environmental samples was observed. Successively, Thung et al. (2018) who found *S. Enteritidis*, and *S. Typhimurium* with prevalence rate of 6.70%, and 2.50%, respectively in raw chicken meat at retail markets in Malaysia. In our study, *S. Typhimurium* was the major serovar which was consistent with the findings of McCrea et al. (2006) who identified *S. Typhimurium* as the major *Salmonella* serovar from a poultry market in California, USA. Saitanu et al. (1994) also reported *S. Typhimurium* (5.5%) was the most predominant serotype in duck eggs in Thailand. The study conducted in Bangladesh revealed *S. Typhimurium* incidence 15.91% in the broiler production systems (Islam et al., 2016); 85% in broiler farm samples (Alam et al., 2020) and 5% in commercial layer farm settings (Parvej et al., 2016). The higher occurrence of *S. Typhimurium* in our study is synchronized with findings at home and abroad. Consecutively, *S. Typhimurium* and *S. Enteritidis* were isolated from raw chicken meat at retail markets in Malaysia (Thung et al., 2018); higher prevalence of *S. Enteritidis* (21.9%) and *S. Typhimurium* (9.4%) were isolated from

chicken in Turkey (Arkali et al., 2020); *Salmonella enterica* serovars were identified in backyard poultry flocks in India (Samanta et al., 2014); *S. Enteritidis* and *S. Typhimurium* recovered from chicken meat in Egypt (Tarabees et al., 2017). Similarly, Suresh et al. (2006) recovered *S. Typhimurium* and *S. Enteritidis* in high proportion compared to other serovars from various poultry products in India. Furthermore, China and some European countries detected *S. Enteritidis* and *S. Typhimurium* as the most prevalent serotypes (Osimani et al., 2016; Zeng et al., 2019).

In our study, NTS *enterica* serovars Typhimurium and Enteritidis along with untyped *Salmonella* were found higher resistance to ciprofloxacin, streptomycin, gentamicin, ampicillin, tetracycline, and nalidixic acid; moderate resistance to sulfamethoxazole-trimethoprim, amoxicillin-clavulanate, chloramphenicol, and azithromycin. Alam et al. (2020) identified a high level of resistance (77.1% to 97.1%) of *Salmonella* isolates to tetracycline, ampicillin, streptomycin, and chloramphenicol. Further, Parvin et al. (2020) reported the highest resistance of *Salmonella* isolates against oxytetracycline (100%), followed by trimethoprim-sulfamethoxazole (89.2%), tetracycline (86.5%), nalidixic acid (83.8%), amoxicillin (74.3%), and pefloxacin (70.3%). Successively, Mridha et al. (2020) demonstrated higher to moderate resistance of *Salmonella* isolates to erythromycin, tetracycline, amoxicillin, and azithromycin. Sequentially, Sobur et al. (2019) found higher resistance of *Salmonella* isolates to tetracycline, ciprofloxacin, and ampicillin. Continually, it has been observed that *Salmonella* originated from fecal samples of chickens, ducks, geese and pigs were resistant to nalidixic acid (48.8%), tetracycline (46.9%), ampicillin (43.2%), streptomycin (38.3%), and trimethoprim/sulfamethoxazole (33.3%) respectively (Long et al., 2016; Im et al., 2015; Vuthy et al., 2017). Concurrently, high resistance to ciprofloxacin (77%), sulfisoxazole (73%) and ampicillin (55.6%) was recorded in chicken hatcheries in China (Xu et al., 2020); tetracycline and ampicillin were found highly resistance in wet markets, Thailand (Sripaurya et al., 2018); higher resistance to nalidixic acid (99.5%), ampicillin (87.8%), tetracycline (51.9%), ciprofloxacin (48.7%), and trimethoprim-sulfamethoxazole (48.1%) were found in broiler chickens along the slaughtering process in China (Zhu et al., 2017); *S. enterica* serovar Typhimurium was found resistant to ampicillin, tetracycline, and sulphamethoxazole isolated from chicken farms in Egypt (El-Sharkawy et al., 2017); high resistance to ampicillin (95.71%), ciprofloxacin (82.86%), tetracycline (100%), and nalidixic acid (98.57%) was observed in isolated NTS at retail chicken meat stores in northern India (Sharma et al., 2019). Furthermore, Siddiky et al. (2021) reported the highest resistance pattern of *S. Typhimurium* isolates to

ciprofloxacin (100%) and streptomycin (100%) followed by tetracycline (86.66%), nalidixic acid (86.66%), gentamicin (86.66%), ampicillin (66.66%) and amoxicillin–clavulanate (40%) in broiler chicken. Sequentially, Siddiky et al. (2021) identified maximum resistance of *S. Enteritidis* isolates to streptomycin (100%) followed by ciprofloxacin (80%), tetracycline (80%), gentamicin (80%), and moderate resistance to amikacin (20%), amoxicillin–clavulanate (20%), azithromycin ((20%), and sulphamethazaxole-trimethoprim (20%) in broiler chicken. There was a very strong correlation and alignment was observed at home and abroad with phenotypic resistance patterns.

In our study, out of seven, a single beta-lactam-resistant *bla*TEM gene was detected in all three types of samples with a diverse prevalence rate. The prevalence of *bla*TEM was detected at 62.06%, 69.62%, and 62.73 in CDW, CBS, and KS. Ahmed et al. (2014) detected a higher prevalence of *bla*TEM gene mediated ESBL production among *Salmonella* isolated from humans in Bangladesh. Yang et al. (2010) identified *bla*TEM, a gene encoded for beta-lactamases resistance, in 51.6% resistant *Salmonella* isolates. Aslam et al. (2012) reported *bla*TEM gene in *Salmonella* isolated from retail meats in Canada with a 17% prevalence rate. Lu et al. (2011) detected only 81.2% *bla*TEM gene, while *bla*CTX-M could not be detected in any of the examined isolates. Similarly, Van et al. (2008) found only the *bla*TEM gene in *E. coli* recovered from raw meat and shellfish in Vietnam. The emergence of *bla*TEM mediated ESBL producing NTS *enterica* serovars indicated the use of beta-lactam antibiotics in poultry farming practices. Siddiky et al. (2021) detected only the *bla*TEM gene from chicken cecal contents and Xiang et al. (2020) reported plasmid-borne and easily transferable *bla*OXA-1 and *bla*TEM-1 genes. Consecutively, Suresh et al. (2019) detected *bla*TEM as the predominant gene in food of animal origin in India. The results of the *bla*TEM gene were in agreement and relevant to previous observations. Successively, in our study, out of three only a single tetracycline resistance gene *tetA* was found with the prevalence rate of 60.32%, 58.92%, and 58.81% in carcass dressing water, chopping board swab, and knife swab respectively. In addition, *sul1*, *sul2*, and *sul3* genes were detected in NTS *enterica* serovars, while *sul1* was predominant. Likewise, streptomycin resistance gene (*strA/B*) was isolated in serovars of NTS with reliable prevalence rate. Arkali and Çetinkaya, (2020) detected 58% positive *sul1* gene from the *Salmonella* isolates of chickens in eastern Turkey. Consequently, Jahantigh et al. (2020) detected the most prevalent *tetA* gene from broiler chicken in Iran. Successively, Vuthy et al. (2017) discovered *bla*TEM, *tetA* and *strA/B* gene from chicken food chain while Sin et al. (2020) isolated *tetA* and *sul1* gene from chicken meat in Korea. Zhu et

al. (2017) who isolated beta lactam (*bla*TEM), tetracycline resistant (*tetA*, *tetB*, *tetC*) and sulfonamide resistant (*sul1*, *sul2* and *sul3*) gene with prevalence at 94.6%, 85.7%, and 97.8% in *Salmonella* isolated from slaughtering process in China. El-Sharkawy et al. (2017) who revealed *bla*TEM, *tetA*, *tetC*, *sul1*, and *sul3* gene from *S. Enteritidis* isolates at a chicken farm in Egypt. Doosti et al. (2016) detected *strA/B* (37.6%) from *S. Typhimurium* isolates at poultry carcasses in Iran. Sharma et al. (2019) detected the most predominant *tetA* and *bla*TEM gene in NTS isolated from retail chicken shops in India. Continually, Alam et al. (2020) revealed *tetA* (97.14%) and *bla*TEM-1 (82.85%) gene in broiler farms whilst Siddiky et al. (2021) detected *tetA*, *sul1*, and *strA/B* gene from chicken cecal contents in Bangladesh. The genotypic resistance patterns were well matched with former findings at home and abroad.

In our study, harmonic and proportioned correlation were existent between genotypic and phenotypic resistance decoration. These findings were in agreement and alignment with the observations of previous study conducted across the globe (Alam et al., 2020; Zhu et al., 2017; Siddiky et al., 2021). However, some disagreement between phenotypic and genotypic resistance pattern might be due to misalignment of antibiotic disk, sensitivity and specificity of disk, primers, concentration of inoculum, laboratory capacity and individual skills (Siddiky et al., 2021). Rather, some research findings supported the misalignment between genotypic and phenotypic resistance patterns (Van et al., 2008; Paterson et al., 2005).

In our study, MDR *Salmonella* embedded mostly with ciprofloxacin, streptomycin, tetracycline, ampicillin, gentamicin and nalidixic acid probably due to common and frequent uses of these antibiotics in poultry production settings in Bangladesh (Al Masud et al., 2020; Parvej et al., 2016). CLSI (2019) reported *Salmonella* became natural resistant to first and second-generation cephalosporin and aminoglycosides. MDR along with higher resistance to ciprofloxacin is very much alarming in human treatment as WHO recommended ciprofloxacin, a first-line drug treatment of intestinal infections. Besides, watch group antibiotic ciprofloxacin had higher resistance and azithromycin had moderate resistance reflects the severity of the resistance pattern of *Salmonella* serovars (WHO, 2019). Furthermore, more resistance to tetracycline indicated the massive use of therapeutic and growth enhancers in poultry production (Wang et al., 2013). The emergence of *bla*TEM mediated ESBL producing *Salmonella enterica* serovars indicated the use of beta-lactam antibiotics in the poultry production cycle. Moreover, ESBL is usually encoded by

large plasmids that are transferable from strain to strain and between bacterial species (Ahmed et al., 2014; González et al., 2013).

Virulence gene analysis indicated *S. Enteritidis* and untyped *Salmonella* isolate carried eight virulence genes including two types of *Salmonella* pathogenicity islands (SPI-1 and SPI-2) and many adhesion-related virulence genes. Virulence genes along with MDR resistance pattern would accelerate the infectivity of *Salmonella* isolates (Huehn et al., 2010). The emergence of antibiotic resistance of *Salmonella* isolates depends on its genetic and pathogenicity mechanisms which can enhance the survivability by preserving drug resistance genes (Aslam et al., 2012). The virulence gene was found more prevalent in *S. Enteritidis* and untyped *Salmonella* isolates compared to *S. Typhimurium* isolates. Six common virulence genes (*invA*, *agfA*, *lpfA*, *hilA*, *sivH*, and *spvC*) were detected in all isolates of *Salmonella* which was incompatible with prior findings around the world (Amini et al., 2010; Borges et al., 2013; Campioni et al., 2012; Crăciuna et al., 2012). Moreover, *sefA* and *spvC* genes were detected in *S. Enteritidis* and untyped *Salmonella* isolates whilst none *S. Typhimurium* isolates carried *sefA* and *spvC* genes. Alike findings have been recorded formerly by researchers (Borges et al., 2013). The higher occurrence of *sefA* in *S. Enteritidis* was compatible with prior findings (Amini et al., 2010; Crăciuna et al., 2012), and *sefA* was somewhat considered target gene to encounter *S. Enteritidis* through the PCR method (Amini et al., 2010). Successively, the *invA* was the most common and virulent gene present in all *Salmonella* isolates and considered as a target gene to identify *Salmonella* species (Nayak et al., 2004; Elkenany et al., 2009). Continually, the *hilA* gene played a key role to exaggerate *Salmonella* virulence by stimulating the expression of invasion into the cell (Ramatla et al., 2017; Cardona-Castro et al., 2002). Furthermore, the virulence gene *invA* and *hilA* could be considered target genes to rapid and reliable detection of *Salmonella* through PCR method. The higher occurrence of *lpfA*, *agfA*, and *sopE* were consistent with previous research results (Collinson et al., 1993; Borsoi et al., 2009). The occurrence of the *sopE* gene (100%) in *S. Enteritidis* was correlated with earlier studies (Batchelor et al., 2005). Further, *agfA* gene liable for biofilm development along with adhesion to cells during infection process (Yoo et al., 2013). In our study, the plasmid-mediated *spvC* virulence gene was detected in *S. Enteritidis* and untyped *Salmonella* isolates which have similarities to earlier observations (Amini et al., 2010; Castilla et al., 2016; Motta et al., 2003). It was previously found *S. Enteritidis* had 92% *spvC* gene while *S. Typhimurium* had only 28% and *S. Hadar* had no (Borges et al., 2013). The higher prevalence of major virulence genes indicated the pathobiology

as well as the public health implications of the serovars. Moreover, all *Salmonella enterica* isolates were found to be highly invasive and enterotoxigenic, which had a significant public health impact. This was the first-ever attempt to determine a wider range of *Salmonella* virulence genes from poultry processing environments in wet markets in Dhaka, Bangladesh. Our results demonstrated that wet markets where chicken has been slaughtered and processed that could spread and harbour NTS *enterica* serovars. Many studies have shown that cross-contamination of poultry could occur during processing and skinning in wet markets due to poor sanitary and hygienic measures (Nidaullah et al., 2017). In wet markets, the sources of contamination may be vendor, chopping board swab, knife swab, carcass dressing water, defeathering machines, scalding water, tanks, floors, drains and work benches etc. (Nidaullah et al., 2017). Free roaming MDR NTS serovars in poultry processing environments could facilitate release into the food chain, agricultural goods, and human populations in wet markets. Furthermore, *Salmonella* serovars were able to survive longer with soil-formed biofilms, and these biofilms were protected from detergents and sanitizers. Therefore, cleaning and sterilization of the knife, chopping board and frequent change of carcass dressing water is crucial to reduce the burden of horizontal transmission of *Salmonella enterica* in wet markets. The root causes indicated that MDR and highly pathogenic NTS *enterica* has been emerged in poultry due to the irrational use of antimicrobials in farming practices.

Conclusion

The higher prevalence of multiple profiles of virulence and multidrug resistance genes of NTS *enterica* serovars in poultry processing environments drew public health attention. The chicken carcass was dressed and processed in open environments in wet markets exacerbated the spread of the pathogen. The unclean and utilized utensils such as chopping boards and knives were used for chicken processing and cutting. Successively, unclean and dirty water were used frequently for chicken carcass dressing and washing. Their numerous risk factors are prevailing at wet markets that can trigger the spread and contamination of NTS serovars. The wet market can be considered as the hotspots of harboring NTS serovars which can easily anchor in the food chain as well as human health. The hidden sources of these MDR pathogens were undoubtedly from chicken which indicated more therapeutic and preventive exposure of antimicrobials during the production cycle. The clean, hygienic and ambient poultry processing environments with good carcass processing practices might reduce the spread and contamination at wet markets. Besides, prudent and

judicious uses of antimicrobials have to be followed in farming practices as poultry farming is considered the fertile ground for uses of antimicrobials. This study could address the potential risk associated with the spread of NTS with multidrug resistance to humans as well as highlight the need of implementing a strict hygiene and sanitation standard in local wet markets.

Supporting Information

S1 Text: Phenotypic and genotypic antimicrobial resistance data

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Fig1

A

Fig2A

(B)

Fig2B

(C)

Fig2C

(A)

Fig3A

(B)

Fig3B

(C)

Fig3C

Fig4