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Table of Content

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Full Length Research Paper

Assessment of antimicrobial resistance in avian pathogenic *Escherichia coli Strains* isolated over four years in Tunisian poultry

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Avian pathogenic *Escherichia coli* strains (APEC) are responsible for major economic losses in poultry farms. The use of antibiotics has led to the emergence of resistant bacteria having direct impact on the food industry. In order to evaluate the resistance of 191 Tunisian APEC strains, we determined the antimicrobial resistance profile of these bacteria to 18 antibiotics by disk diffusion method. This study revealed high resistance towards most of the tested antibiotics. Indeed for 13 antibiotics over 50% of strains were resistant. The results also showed significant increase in time of resistance percentage and multidrug resistance; which may be related to the selection pressure due to the overuse of antimicrobial agents for treatment and as growth factors in poultry. Statistical tests revealed several statistical descriptive values, reflecting scattered distribution of resistance with normality dispersion. Phylogenetic analyses showered clustered strains. Data converge towards a heterogeneous distribution of resistance with increasing rates, suggesting considerable overlap between APEC strains.

Key words: APEC strains, colibacillosis, antimicrobial agents, resistance profile, Tunisia.

INTRODUCTION

The poultry industries worldwide suffer great financial losses every year because of the high morbidity and mortality rates caused by colibacillosis, common bacterial infection (Guerin and Boissieu, 2008) are mostly important in avian pathology. Colibacillosis is caused by avian pathogenic *Escherichia coli* (APEC) (Lau et al., 2010; Oh et al., 2011) with a broad spectrum of clinical outcomes. APEC strains are endowed with different properties that allow them, for example: to enter the bloodstream, overcome to host defense mechanisms or colonize deep organs and is a subset of extra intestinal pathogenic *E. coli* (ExPEC) (de Pace et al., 2011). They share virulence traits with strains isolated from human cases of neonatal meningitis, urinary tract infections, and septicemia. Thus, APEC strains represent a high risk of zoonotic infection (Bauchart et al., 2010) and their virulence gene pool may contribute to the emergence of other ExPEC strains (Bertrand et al., 2010). Avian

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colibacillosis takes various shapes with a general process of respiratory or genital input (Bertrand et al., 2010) and can affect digestive, biliary and urinary tracts, which are major source of contamination in poultry farms (Oyetunde et al., 1978). APEC strains commonly cause airsacculitis, pericarditis, perihepatitis, peritonitis, salpingitis, and subsequently the most acute form, septicemia, resulting in sudden death (Mellata et al., 2003; Ask et al., 2006; Zhao et al., 2009 ; Giovanardi et al., 2013). It has been shown that from 10 to 15% colibacillary population belongs to potentially pathogenic serotypes (Dho-Moulin and Fairbrother, 1999).

In Tunisia the poultry industry is an important part of the economy and treatment strategies which are based on the use of antibiotics and control environmental factors. Antimicrobial treatments against colibacillosis are usually given to the whole flock via the drinking water or feed over several days and thus may impact the equilibrium and susceptibility of bacteria present in the intestinal flora. The poorly controlled use of broad-spectrum antibiotics has favored the emergence of highly resistant bacteria, which place the treatment of certain infections in therapeutic impasses. The acquired resistance of APEC strains to several antimicrobial drugs is becoming a major issue in intensive poultry farming (Furtula et al., 2010). Furthermore, the risk of consuming chicken meat contaminated with resistant *E. coli* consists mainly of the possible transfer of resistance genes to other, potential pathogenic bacteria, present in the human intestinal tract (Markland et al., 2015).

The increasing incidence of antibiotic resistance in APEC strains and the high risk of transmission to humans and potential effect on the environment, especially because litter from farms is commonly used as fertilizer, is an area of growing concern (Furtula et al. 2010). The purpose of this study was to investigate antibiotic resistance of APEC strains isolated at the Veterinary Research Institute of Tunisia (IRVT) from poultry in Tunisian commercial poultry farms in order to study resistance dynamics and transfer. This may give new insight in improving treatment strategies. The focused on this study is on 18 antimicrobials, administered over four years.

MATERIALS AND METHODS

Isolation of *E. coli* strains

A total of 191 APEC strains were collected over a four-year period (from April 2010 till April 2014) from Tunisian poultry and isolated from different organs (livers, hearts and spleens) of sick chickens exhibiting clinical symptoms of avian colibacillosis in «diagnostic bacteriology laboratory of the Veterinary Research Institute of Tunisia». Number of strains and sites of isolation are depicted in Table 1.

Table 1. Number of strains and corresponding site of isolation.

Number of strains	Site of isolation
128	Livers, Spleens
63	Livers, Spleens, Hearts

For 128 strains, samples were isolated from living organs: livers and spleens at the same time For 63 strains samples were isolated from living organs: livers, spleens and heart at the same time All tests were performed aseptically to avoid contamination by non-pathogenic bacteria.

Growth conditions of APEC strains

The samples, which were collected from affected organs, were grown in Bromocresol Purple Lactose Agar (BCP) medium aerobically for 18 to 24 h at 35 to 37°C. Specimens must be directly streaked onto the medium not later than 2 h after collection or must be kept refrigerated (not longer than 24 h) to avoid overgrowth of the infectious agents or contaminants. Differentiation of APEC isolates from other specimens was performed by Gram stain followed by appropriate standard biochemical tests (oxidase test, urease, B-galactosidase, Kligler iron agar, citrate permease etc) and commercial API 20E antisera test according to the manufacturers' instructions (Biomérieux).

Antibiotic susceptibility testing assay

Several assays for estimating antimicrobial susceptibility of 18 antibiotics belonging to most known families with direct interest to human health were conducted for the 191 APEC strains using the disk diffusion method recommended by Antibiogram Committee of the French Society for Microbiology CA-SFM, according to the French Veterinary Benchmark Standards (Haenni et al., 2011). Mueller Hinton agar plates were inoculated with an inoculum of *E. coli* strains and disks impregnated with antimicrobial agents were filed on the inoculated agar plates. After incubation at 37°C for 18 to 24 h, the study of the bacteriostatic effect of antibiotics was determined by measuring the diameter of the inhibition zone around the disk. Details regarding families of antibiotics tested are listed in Table 2.

Reference strains (E. coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853) were used as susceptibility testing quality control in order to ensure the validity of the results.

Statistical analysis

Statistical analyses were performed with R programming language and software environment for statistical computing and graphics (version3.0.2). R functions are executable through command lines and scripts. Our data were analyzed using statistical tests of R package that calculate resistance rates to antibiotics in APEC strains and determine significant differences between them. We also determined several statistical descriptive measures such as variance and SD (deviation) of resistance dispersion. The Shapiro-Normality test was used to analyze the normality of resistance distribution. This test is based on *W* statistic that offers a *w* value associated to *P*-value. A *P*-value less than 0.05 are considered statistically significant supporting that the resistance does not follow a normal distribution.

Phylogenetic analysis

Phylogenetic analysis was performed with R program in order to

Table 2. List of antibiotics tested associated to their families.

Family	Subfamily	Group	Antibiotics or chemotherapeutic agent
Beta-Lactam	Penicillin (Penams)	Group A	Ampicillin (AM)
			Amoxicillin (AMX)
			Amoxicillin-clavulanic acid (AMC)
	Cephalosporins (cephams)	First generation	Cephalexin (CN)
			Cefalotin (CF)
		Second generation	Cefoxitin (FOX)
		Third generation	Ceftiofur (XNL)
Aminoglycosides			Streptomycin (STR or S)
			Neomycin (N)
			Gentamicin (GM)
			Spectinomycin (SPT)
Tetracyclines	First generation		Tetracycline (TE)
Polypeptides	Overactive (detergents)		Colistin (Polymyxin E) (CS50)
Quinolones	First generation		Nalidixic acid (NA)
	Second generation		Flumequine (UB)
	Third generation (fluoroquinolones)		Enrofloxacin (ENR)
			Marbofloxacin (MAR)
diaminopyrimidines			Trimethoprim-sulfamethoxazole (SXT)

test phylogenetic links between resistant strains. Input data were translated from excel table to a matrix in binary format to be correctly treated by R commands.

RESULTS

Assessment of antibiotic resistance rate

The results of resistance testing to all antibiotics showed variable rates ranging from 8% (intermediate resistance level) of strains resistant to ceftiofur (XNL) to 86% (high resistance level) of strains resistant to tetracycline (TE). Among strains tested, more than 50% exhibit resistance to 13 of the 18 tested antibiotics, ampicillin, amoxicillin, amoxicillin-clavulanic acid, cephalexin, streptomycin, neomycin, spectinomycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, flumequine, enrofloxacin and marbofloxacin. For the other five antibiotics the rate of resistant strains was comprised between 8% and 39%. Thus, for the majority of samples resistance rate was described as high. Resistance and susceptibility rates for all antibiotics are plotted as histograms and depicted in Figure 1.

Comparison between antibiotic resistances in different periods of time was conducted and an increase in number of resistant APEC strains from a year to another was noticed with the highest global level in 2013. For nine antibiotics: cephalexin, cefalotin, cefoxitin, ceftiofur, neomycin, spectinomycin, trimethoprim-sulfamethoxazole, enrofloxacin and marbofloxacin has the highest resistance level and was observed in 2013; for five antibiotics: ampicillin, amoxicillin, tetracycline, colistin and flumequine, has the highest resistance level which was observed in 2010; for two antibiotics: amoxicillin-clavulanic acid and nalidixic acid, resistance was the highest in year 2011 and 2012, respectively, and for the two latest antibiotics: the highest level was observed once in both 2010 and 2012 (for streptomycin) and again in both 2011 and 2013 (for gentamicin). A slight decrease in global resistance rate was observed in 2014. Moreover, it seems that this spread does not depend on the families of antibiotics tested. Indeed, antibiotics belonging to the same family could be prevalent each in different period. Only one case of extremely related antibiotics those of the cephalosporin subfamily (Beta-lactam family), predominant in 2013 with four patterns, was observed. These findings are summarized in Figure 2.

Statistical tests were performed with R language software and revealed several statistical descriptive values informing on distribution and correlation between variables characterizing evolutionary trends of APEC strains drug resistance. The average of resistance rate for all antibiotics, obtained by dividing the sum of all resistant rates by their number, was 67.83 with a standard deviation of 43.57 (67.83±43.57). The coefficient of variation (cv) representing the dispersion of drug

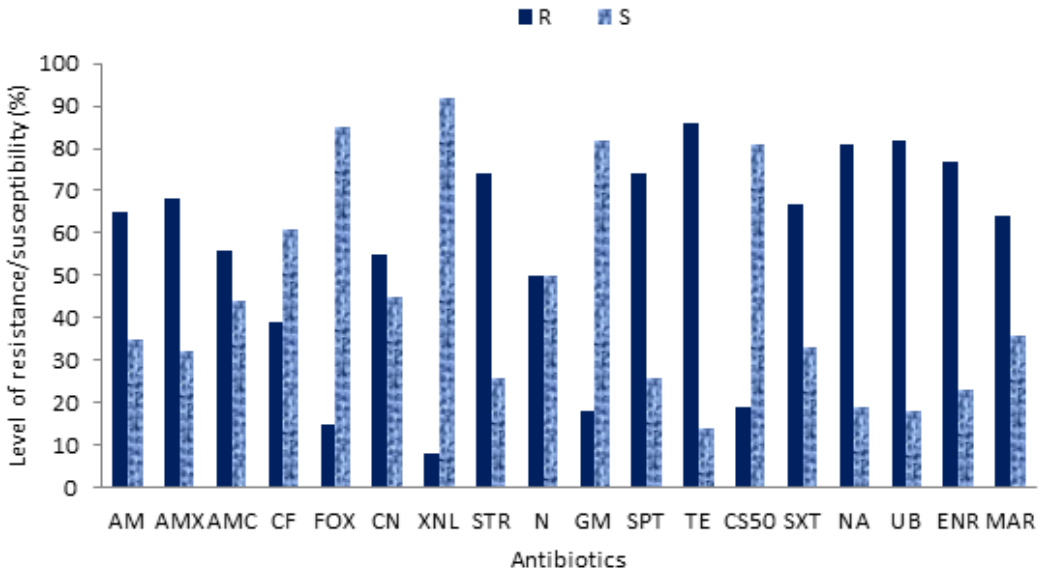

Figure 1. Resistance and susceptibility levels of APEC isolates to antibiotics of interest.

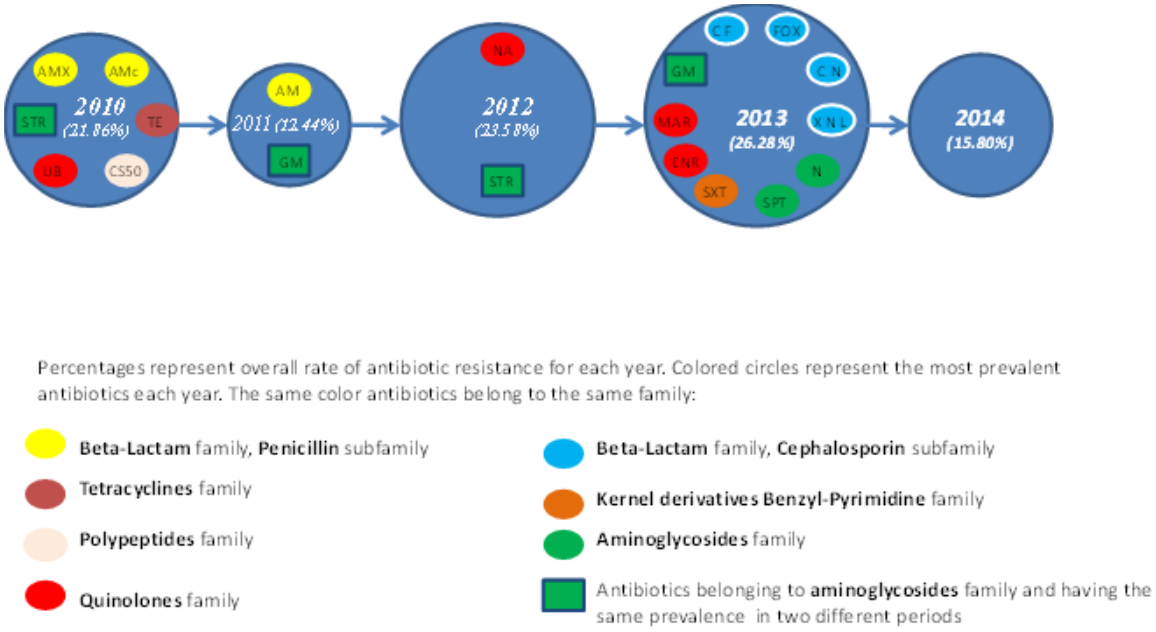

Figure 2. Evolution of overall drug resistance in APEC strains during the study period.

resistance rates versus the average value, was also determined (64.23) and showed that the variation of resistance to all antibiotics tested, tended to be scattered as compared to their average, meaning that different drug resistant rates were statistically distant since cv > 50. *W* and *P*-values were also determined to study the nature of different antibiotics resistance distribution; *w* = 0.93 and *P*-value = 0.24. Thus, the distribution of resistance was normal since *P*-value was found to be > 0.05.

Most isolates exhibit multidrug resistance

Among the 191 isolates studied, 168 (88%) specimens were resistant to at least three antibiotics at the same time and so have multidrug resistance profiles. Whereas, the other 23 strains (12%) were resistant to one or two antibiotics each. These results showed an increase in multidrug pathogenic *E. coli* that could be related to the overuse of antibiotics in the veterinary field. In fact, it has

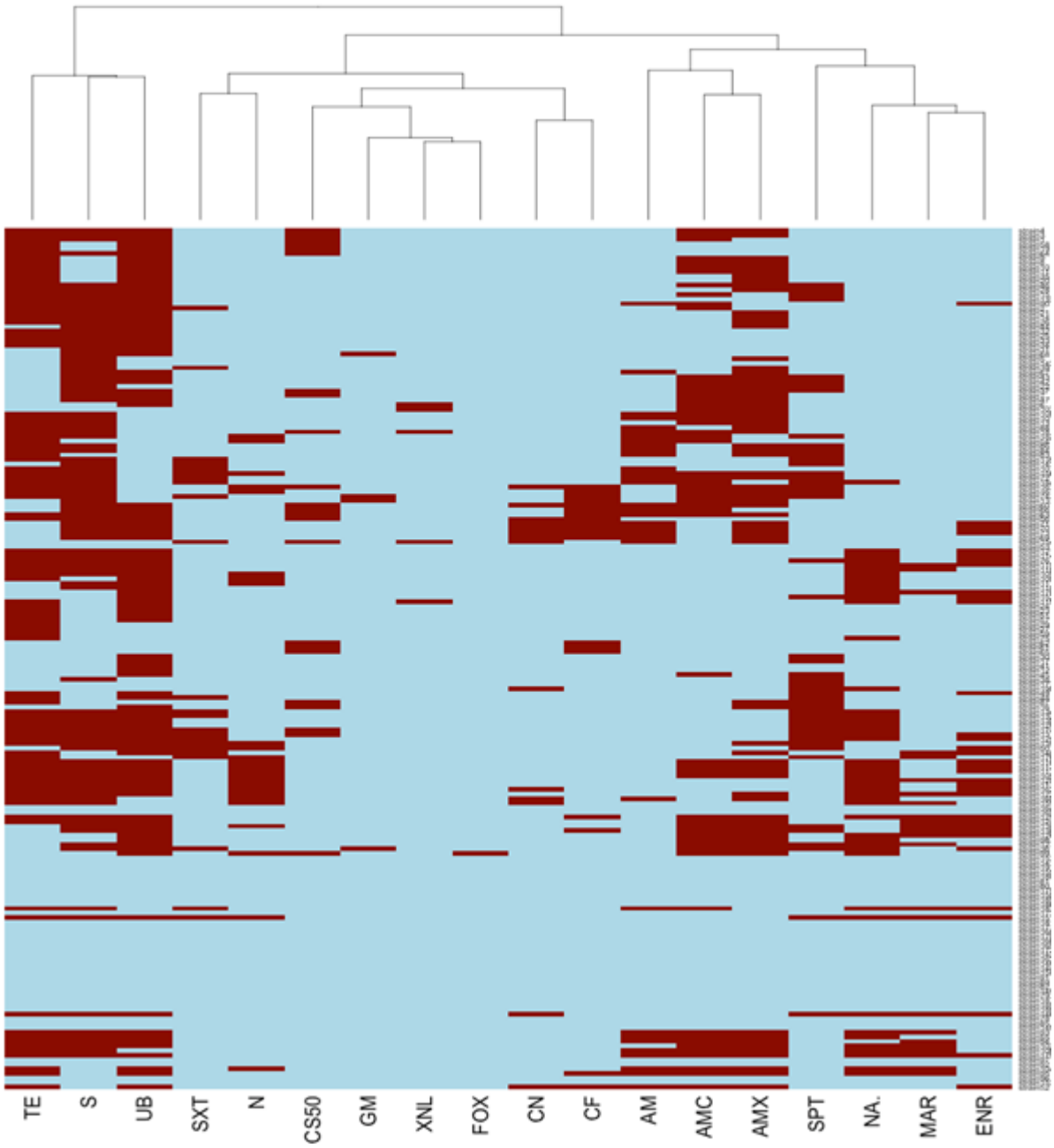

Figure 3. Hierarchical clustering rate based on antibiotics studied.

been shown that the use of antimicrobial agents is associated with antimicrobial resistance and even leads to human health consequences (Anqulo et al., 2004; Zhao et al., 2012).

Phylogenetic construction and clustering rate

Phylogenetic analyses were conducted with R programming software in order to determine relatedness link between isolates on the base of resistance that exhibit different antibiotics. Phylogenetic tree was constructed and visualized with distance matrix method. Comparing drug resistance profiles strains were subject to our phylogenetic study which revealed the presence of twenty-two clusters, fourteen clusters which composed of two strains, five clusters composed of three strains, two clusters composed of four strains and finally one cluster composed of five strains showing the same drug resistance profile (Figure 3). Clusters were mainly associated to antibiotics for which, the resistance was high.

DISCUSSION

In the present study we explored the resistance of pathogenic *E. coli* strains isolated from Tunisian poultry to 18 antimicrobial agents belonging to the most common antibiotics families used in Tunisia to treat avian colibacillosis. This has been achieved according to the standards adopted by the French Society for Microbiology Committee (Haenni et al., 2011). For this purpose, we focused on the study of 191 isolates.

Based on the resistance profile, we noted that highly polymorphous resistance rates have been displayed for one antibiotic to another with global high level in most cases studied. The level of resistance to ampicillin observed in our study (65%) was rising continuously which is consistent with results previously reported. A previous work conducted in diagnostic bacteriology laboratory of IRVT revealed ampicillin resistance rate which is close to our findings (52.5%) (Data not published). Another study conducted by Zhao et al (Zhao et al., 2012) showed an increase in ampicillin resistance over time. Similar resistance profiles to amoxicillin and amoxicillin-clavulanic acid were observed which seems to be quite expected seeing that these antimicrobials were widely used in various respiratory infections treatment (Gaillat et al., 1987). Resistance to nalidixic acid was also high (81%) which could be explained by cross-resistance with that to oxolinic acid as they have the same regulating functional role of blocking the same enzymes during DNA synthesis.

As regular monitoring of antibiotic resistance is a key to effective and appropriate therapeutic strategies limiting the emergence and the spread of multidrug-resistant strains, we looked for the antibiotic resistance combination exhibited by each strain. Our data showed the grouping of strains in clustered profiles suggesting horizontal transfer of antibiotic resistance.

Taken together, our data converge towards a heterogeneous distribution of resistance with increasing rates that revealed a considerable overlap between APEC strains reminding clonal expansion. In fact, resistance genes are known to spread via two phenomena, horizontal gene transfer and clonal expansion. Such variability seems reasonable as a result of the overuse of antibiotics in the treatment of colibacillosis, a treatment that is sometimes inappropriate and not controlled when administrated in poultry farms (Salehi and Bonad, 2006).

It has been previously shown that genes encoding antibiotic resistance are commonly found in *E. coli* from different hosts (Venturini et al., 2013). Thus, APEC strains probably serve as a reservoir of genes encoding resistance proteins which could explain the rapid dissemination of antibiotic resistance. On the other hand, the use of antimicrobial agents as growth promoters in poultry feed has an important implication on the emergence of antimicrobial resistance in bacteria (Smith et al., 1999; Shuford and Patel., 2005). Fortunately this practice is banned in Tunisia since 2007. However, the misuse of these antimicrobials preventively remains a concern. Combination of these antimicrobials and resistant *E. coli* could be risk factor for environmental contamination that could be transferred to human. In fact, it has been shown that the same type of *E. coli* carrying an identical gene encoding sulphonamide resistance (*sul2*) can colonize both animals and humans, and that strains which can be found among animals which may be implicated in human infections such as septicaemia (Trobos et al., 2009). APECs probably serve as source of human infection by pathogenic *E. coli* through transmission via the food chain (Zhao et al., 2009) of several known drug resistance genes (de Pace et al., 2011), such as those encoding siderophores and capsules. Thus, zoonotic potential of animal-derived strains need to be more explored specially with increasing knowledge of molecular genetics and pathotypes of ExPEC of human and animal origin. Controversy continues to be needed to determine the pathogenicity of APEC strains as well as the potential effect of antimicrobial residues analysis on public health.

Conclusion

Since infections referred to avian colibacillosis are responsible for large financial losses to the poultry industry each year due to mortality, lost production and condemnations, antimicrobial treatment has become a common practice which has however several implications affecting the poultry sector mainly in relation to the emergence of antibiotic resistant strains. In this study, we plotted the epidemiological distribution and evolution of antimicrobial resistance dynamics in Tunisian APEC strains that evolve exponentially during these last few years. It has been concluded that, such epidemiological studies provide effective tools in antibiotic resistance studies; nevertheless further work is needed to define additional biological features.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Emerging threats of antibiotic resistance in *Salmonella typhi* and *Salmonella paratyphi A* among enteric fever cases of Dhaka, Bangladesh

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Enteric fever is a severe public health threat because of the rising antibiotic resistance of *Salmonella* species in developing countries, especially in its endemic areas like Bangladesh. This retrospective study was aimed to assess the effectiveness of a range of 17 commonly used antimicrobials against *Salmonella* Typhi and *Salmonella Paratyphi A* isolated from 601 enteric fever cases in Dhaka, Bangladesh. Conventional biochemical tests were used to identify *Salmonella* strains and the Kirby-Bauer disc diffusion method to perform the antibiotic sensitivity in SAIC Digital Diagnostic Lab, Dhaka. The 2017 Clinical Laboratory Standard Institute (CLSI) guideline was employed to interpret the antibiogram results, and statistical software SPSS (version 22.0) to analyze the obtained data. The number of male patients (54.74%) dominated over their female counterparts (45.26%). The patients aged from 1 month to 75 years, with a mean of 19.74±12.79 years. Among 601 *Salmonella* spp. isolates, *S.* Typhi (56.57%) prevailed over *S.* Paratyphi *A* (43.42%). Both strains showed >85% antimicrobial insusceptibility to three major antibiotics: ciprofloxacin, gentamicin, and amikacin. *S.* Typhi (65.29%) showed significantly greater resistance to azithromycin compared to *S.* Paratyhi *A* (14.9%) (p<0.001). Both pathogens reported over 95% sensitivity to ceftriaxone, cefixime, ceftazidime, amoxiclav, cephalexin, aztreonam, imipenem, and cefuroxime. To conclude, this study found an increased antibiotic resistance of *Salmonella* spp. to commonly prescribed antibiotics. These findings would help physicians and policymakers make informed decisions and provide better treatment to the affected patients.

Key words: *Salmonella*, antimicrobials, antimicrobial insusceptibility, antibiotic sensitivity, Dhaka.

INTRODUCTION

Enteric fever is a life-threatening systemic illness caused by Gram-negative *Salmonella* Typhi and *Salmonella* Paratyphi *A* (Crump and Mintz, 2010). It attacks almost 16 million people each year and causes over 153,000

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deaths worldwide; notably, most of them belong to South Asia and sub-Saharan Africa. In 2017, nearly 17 million people worldwide got infected, and 117,000 patients lost their valuable lives with a heightened mortality of 4 to 5% (Global Burden of Disease Study, 2017). Its widespread prevalence in the developing and tropical regions like Asia and Africa is primarily due to the existing inadequate food and water safety. Likewise, this contagious fatal disease has also become endemic in Bangladesh (Crump and Mintz, 2010; Kirk et al., 2015). Between 2003 and 2004, Bangladesh reported enteric fever incidence as 200 episodes per 100,000 individuals each year compared to 394.2 episodes per 100,000 individuals in South Asia (Saha et al., 2018). One recent study by Ahmed et al. (2017) explored the bacterial etiology of bloodstream infections and found *S.* Typhi and *S.* Paratyphi *A* as the most frequently isolated organism with a high percentage of multidrug-resistant (MDR) strains (Ahmed et al., 2017). Worryingly, younger children in Bangladesh have experienced the highest incidence of enteric fever compared to Vietnam and other comparable regions (Brooks et al., 2005).

This deadly infection is regarded as “typhoid” when caused by *S.* Typhi and “paratyphoid” fever when by *S.* Paratyphi. These pathogens can transmit through the oral or fecal routes of patients and manifest morbidity through multiple signs: fever, abdominal pain, and non-specific symptoms, including nausea, vomiting, headache, and anorexia (Connor and Schwartz, 2005; Sur et al., 2007). When ingested, these *Salmonella* species bacteria colonize the small and large intestines, invade the gastrointestinal barrier, and then spread to the vital organs such as the liver, spleen and bone marrow (Raffatellu et al., 2008). However, due to increasing resistance of *S.* Typhi, the available antibiotics that can be considered for effective treatment are decreasing day by day (Das et al., 2017; Saha et al., 1997). This situation has been deteriorating abruptly in low and middle-income countries because of the higher antimicrobial resistance of *S.* Typhi and *S.* Paratyphi *A* strains. Multiple factors like incomplete treatment, overuse, and over-the-counter sales of antibiotics may contribute to this public health concern of antimicrobial resistance. Several studies confirmed that *S.* Typhi was first reported MDR against ampicillin, chloramphenicol, and cotrimoxazole in the early 1970s and ciprofloxacin in the early 1990s (Olarte and Galindo, 1973). Nowadays, roughly 90% clinical isolates from the urban settings of endemic regions showed decreased sensitivity to ciprofloxacin (Das et al., 2017; Iyer et al., 2017). Later, this trend also shifted to other classes of antibiotics such as azithromycin and ceftriaxone (Das et al., 2017). A recent study from Pakistan also revealed that S. Typhi induced extensive drug-resistance to ciprofloxacin and ceftriaxone (Klemm et al., 2018). Therefore, this study was carried out to investigate the current antibiotic susceptibility patterns of *S.* Typhi and S. Paratyphi *A*. Its findings would benefit healthcare professionals in making informed decisions and providing better treatment for enteric fever patients in the coming days.

METHODOLOGY

Study design and setting

A retrospective study spanning approximately one year (January 2019 to November 2019) was conducted based on the laboratory records of the SAIC Digital Diagnostic Lab database, Dhaka. In total, 601 blood culture-positive samples collected from the enteric fever patients were assigned for the study. A semi-structured checklist was used to extract all cultures and antimicrobial sensitivity test results of patients from the laboratory records notebook.

Isolation and identification of *Salmonella* spp.

Gram-staining and conventional biochemical methods were used to identify the *Salmonella* isolates (Figure 1). A culture media enriched with Selenite broth was used to support the likely growth of pathogens (Figure 2). Following the inoculation, the media was incubated overnight at 37°C and sub-cultured into *Salmonella-Shigella* agar, blood agar, and Mac-Conkey agar. Triple sugar iron (TSI) agar was initially used to differentiate the isolated *Salmonella* strains, resulting in alkaline slant, acidic butt, and H_2S production. *S.* Typhi produced H_2S but not gas, whereas *S.* Paratyphi *A* produced gas and some *S.* Paratyphi *A* produced H_2S after 72 h. Both strains were motile but showed negative reactions in indole, citrate, and urea tests.

Antimicrobial susceptibility test (AST)

To determine the antibiotic susceptibility of *Salmonella* isolates, the Kirby-Bauer disc-diffusion method was performed on Muller-Hinton agar plates shown in Figure 2, (Bauer et al., 1966). Antibiotics used were selected based on their 2017 Clinical Laboratory Standard Institute (CLSI) guideline (CLSI, 2017), local prescription by physicians, and availability in the market. All isolates were tested against 17 different types of antibiotics from 8 classes: β–lactamases (Ampicillin-10 µg, Aztreonam-30 µg, Amoxicillin-Clavulanic acid- 30 µg), Carbapenem (Imepenem-10 µg), Aminoglycosides (Gentamycin-10 µg, Amikacin-30 µg), Co-trimoxazole (Co- trimoxazole-25 µg), Cephalosporin (Cefepime 30 µg, Ceftriaxon 30 µg, Cefixime 5 µg, Ceftazidime 30 µg, Piperacillin 75 µg, Cephalexin 30 µg, Cefuroxime 30 µg), Fluoroquinolone (Ciprofloxacin 5 µg), Tetracycline (Tetracycline-30 µg), and Macrolide (Azithromycin-10 µg). Subsequently, the results of AST were interpreted according to the CLSI 2017 guideline.

Statistical analysis

The data were tabulated and illustrated graphically using Microsoft Excel-2019 and subsequently analyzed by the statistical software, SPSS-22. The descriptive results were represented as a percentage, relative frequency, mean ± standard deviation (SD). At last, to find the association between the types of *Salmonella* spp. infection with patients’ attributes, and antibiotic sensitivity against the tested antibiotics, Chi-square tests and Independent Sample t-test were applied.

Ethical considerations

The Institutional Review Board and chairperson of the SAIC Digital

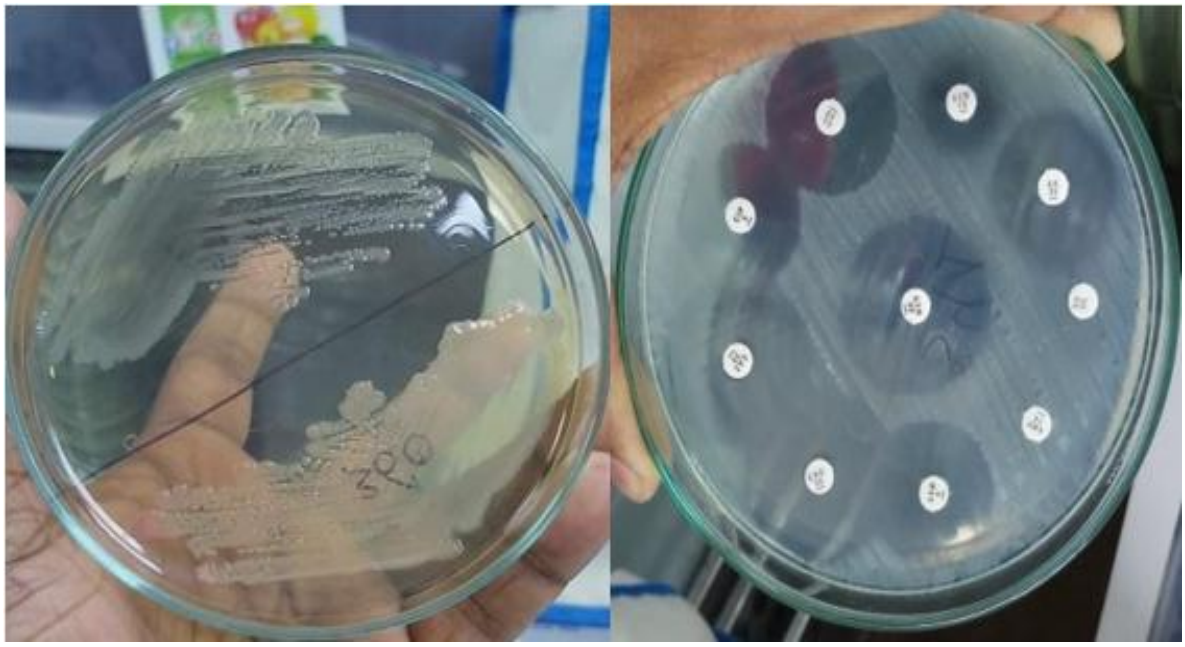

Figure 1. Biochemical tests.

Figure 2. Culture and sensitivity tests.

Diagnostic Lab, Dhaka, acknowledged the required ethical approval for the study. It was ensured that the patients selected for the study had not received any antibiotics before 8 h of their sample collection.

Table 1. Distributions of positive cases based on sex and age of the patients.

Patients' sex and age	*Salmonella* spp.		Total [n (%)]	Statistical Tests
	Salmonella Paratyphi *A* [n (%)]	*Salmonella* Typhi [n (%)]		
Sex of the patients				
Male	145 (44.1)	184 (55.9)	329 (54.74)	$\chi2$=0.123; p=0.726
Female	116 (42.6)	156 (57.4)	272 (45.26)	
Total n (%)	261 (43.42)	340 (56.57)	601 (100)	
Age of the patients (years)				
<5	31 (47.7)	34 (52.3)	65 (10.82)	$\chi2$=6.184; p=0.186
5-20	115 (40.4)	170 (59.6)	285 (47.42)	
21-40	104 (48.4)	111(51.6)	215 (35.77)	
41-60	8 (29.6)	19 (70.4)	27 (4.49)	
>60	3 (33.3)	6 (66.7)	9 (1.50)	
Mean±SD of age (years)	19.87±11.97	19.64±13.39	19.74±12.79	t=0.218, p=0.827
Median age (years)	19.00	17.00	18.00	
Range of age	-	-	1 month to 75 years	

$\chi2$=Chi-square Value, p=significance value at LS = 0.05.

RESULTS

Among 601 *Salmonella* isolates, 340 (56.57%) and 261 (43.42%) were confirmed as *S.* Typhi and *S.* Paratyphi *A*, respectively. The number of male patients (54.74%) predominated their female counterparts (45.26%). But, the distribution of male and female patients based on their infections either by *S.* Typhi or *S.* Paratyphi *A* was similar (p>0.05). Males and females suffered more from S. Typhi than *S.* Paratyphi *A*; about 60% males and 57% females tested positive for *S.* Typhi. The patients aged from 1 month to 75 years, with a mean of 19.74±12.79 years. The average age of the patients infected by *S.* Typhi and *S.* Paratyphi *A* was nearly the same: 19.64±13.39, and 19.87±11.97 years, respectively. The majority of the patients, almost 83%, were 5-40 years old. Patients of the 5-20 years group accounted for the highest, 47.42%, among all enteric fever cases, followed by the adult group, 21-40 years, contributing to 35.77% enteric fever cases. The least number of patients (1.5%) belonged to the oldest age group, >60 years. When *S.* Typhi and *S.* Paratyphi cases were distributed within different age groups, the number of typhoid patients outnumbered the paratyphoid patients in each age group. Within the groups of 41-60 and >60 years, the typhoid patients nearly doubled that of paratyphoid. The infection by both pathogens was most common among the age groups of 5 to 20 years, followed by 21-40 years (Table 1).

As shown in Figure 3, S. typhi and S. paratyphi A were highly insensitive (>85%) to ciprofloxacin, gentamycin, and amikacin. On the other hand, nearly 10-20% cases by both pathogens had developed resistance to cotrimoxazole, piperacillin, and ampicillin. Interestingly, 5 out of 17 antimicrobials tested showed invariable efficacy against nearly all typhoid and paratyphoid cases: cefixime, ceftazidime, cephalexin, aztreonam, and amoxicillin. Ten antibiotics were highly sensitive against *S.* Typhi; they all showed over 95% susceptibility (ceftriaxone 99.71%, ceftazidime 99.71%, cefepime 99.65%, cefixime 99.41%, cephalexin 98.51%, cefuroxime 98.23%, imipenem 97.35%, amoxiclav 397.31%, aztreonam 97.30% and tetracycline 96.51%). In striking resemblance with *S.* Typhi, 8 out of those 10 antimicrobials had over 95% efficacy against *S.* Paratyphi *A* as follows: ceftriaxone 99.2%, cefixime 98.9%, ceftazidime 98.9%, amoxiclav 98.1%, cephalexin 97.7%, aztreonam 96.5%, imipenem 96.2%, and cefuroxime 96.2%. On the other hand, *S.* Typhi demonstrated as high as over 85% resistance to the following antibiotics (gentamycin 99.12%, amikacin 99.41%, and ciprofloxacin 85.50%); however, *S.* Typhi showed lower resistance against other remaining antimicrobials (azithromycin 65.29%, cotrimoxazole 22.65%, piperacillin 21.32%, and ampicillin 19.53%) (Table 2).

Similar to the resistance shown by S. typhi, S. paratyphi A was found to be sensitive to cefepime 83.1%, tetracycline 93.5%, cotrimoxazole 87.3%, piperacillin 86.4%, and amikacin 91.9%. Likewise, *S.* Paratyphi *A* too showed over 85% insensitivity to the antibiotics (gentamycin 99.1%, amikacin 98.5%, and ciprofloxacin 88.5%, followed by cotrimoxazole 12.7%, piperacillin 13.6%, and azithromycin 14.9%) (Table 2). When the sensitivity of each antibiotic was distributed against the type of *Salmonella* spp., several significant variations (p<0.05) were observed in their sensitivity. Cefepime showed significantly uneven resistance to *S.* Typhi (.35%) and *S.* Paratyphi *A* (16.9%) (p<0.001).

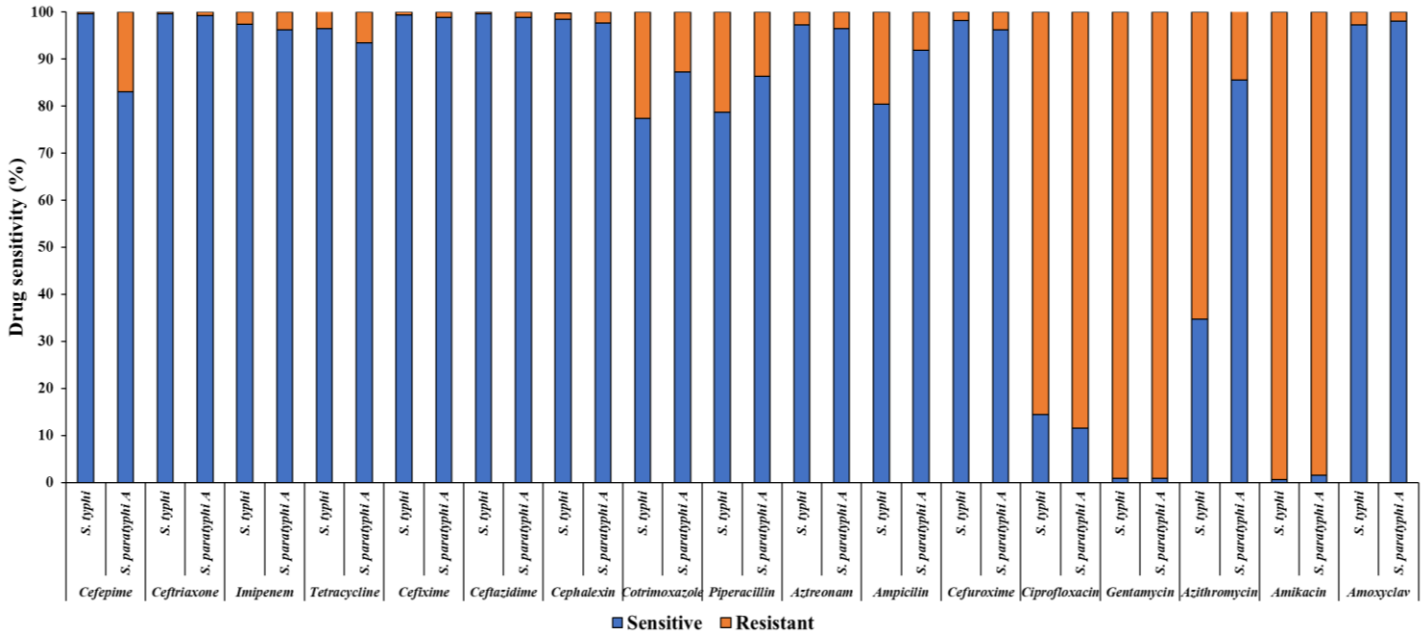

Figure 3. Simplified graphical presentation of antibiotic sensitivity of S. Typhi and S. Paratyphi A.

Cotrimoxazole was two times more resistant against *S.* Typhi (22.65%) compared to *S.* Paratyphi *A* (12.7%) (p=0.002). *S.* Typhi (19.53%) showed almost double insensitivity to ampicillin compared to *S.* Paratyphi *A* (8.1%) (p<0.001). Overwhelmingly, *S.* Typhi (65.29%) was about five times more resistant to azithromycin than *S.* Paratyphi *A* (14.9%) (p<0.001).

DISCUSSION

Enteric fever is a growing public health concern in developing and tropical countries, including Bangladesh. The indiscriminate use of antibiotics has intensified the problem by converting the previously sensitive drugs into resistant ones against the causative agent, *Salmonella* spp. In the present study, the existing susceptibility of *S.* Typhi and *S.* Paratyphi *A* were tried to investigate against some common antibiotics used to treat enteric fever.

This study showed, *S.* Typhi affected more enteric fever cases slightly compared to *S.* Paratyphi *A*, which is consistent with a previous study conducted by Ahmed et al. (2017). Likewise, Raza et al. (2012) also found that 55.8% of the enteric fever cases were diseased by *S.* Typhi and 44.2% with *S.* Paratyphi *A*. However, *S.* Typhi (66.6%) affected the number of enteric fever patients two times more than *S.* Paratyphi *A* (33.3%) (Guha et al., 2005). As far as the number of patients infected by both *Salmonella* infections, male patients dominated the females, with a proportion of 1.20:1. Accordingly, several studies presented that males were increasingly more susceptible to *Salmonella* spp. over females (Chowta and Chowta, 2005; Kumar et al., 2008).

In this study, patients aged 5-20 years accounted for the maximum enteric fever cases, whereas children under-5 years were less vulnerable than their older peers. Likewise, an earlier study revealed the majority of selected patients (63.8%) were 6-15 years, followed by the 16-25 years age group (22.41%) (Sattar et al., 2017). Again, Brooks et al. (2005) found that above-5 years children were more susceptible to enteric fever than those under-5 years, which is also comparable to our findings. Under-5-year cases, in this study, had slightly more chance to be affected by typhoid relative to paratyphoid fever. Some studies also found under-5-year children were more frequently affected by typhoid in comparison with paratyphoid fever (Naheed et al., 2010; Sinha et al., 1999). Although, some studies suggested that young children are less prone to typhoid fever (Ferreccio et al., 1984; Khanam et al., 2015).

In this study, *S.* Typhi was highly sensitive to cefepime, ceftriaxone, tetracycline cefixime, ceftazidime, cephalexin, cotrimoxazole, piperacillin, aztreonam, amoxiclav, and cefuroxime. Similarly, Ahmed et al. (2019) showed *Salmonella* spp. was highly effective against cefixime and ceftriaxone (Ahmed et al., 2019). Greater sensitivity of ceftriaxone to *S.* Typhi was also earlier found by another study (Britto et al., 2018). But, in sheer contrast to ours finding, a relevant Bangladeshi study in 2015 found higher resistance of *S.* Typhi for cotrimoxazole, cefixime, tetracycline, and ceftriaxone (Rahman, 2015). *S.* Typhi was highly sensitive to imipenem. Accordingly, imipenem (carbapenem) maintained high sensitivity to *S.* Typhi in many past studies. Rahman et al. (2015) reported

Table 2. Patterns of antibiotic sensitivity of both *Salmonella*.

Antibiotics	Sensitivity	*Salmonella* spp.		Chi-square	p
		Salmonella Typhi [n (%)]	*Salmonella* Paratyphi *A* [n (%)]		
Cefepime	S	285 (99.65)	217 (83.1)	49.20	**<0.001****
	R	1 (0.35)	44 (16.9)		
Ceftriaxone	S	339 (99.71)	259 (99.2)	0.663	0.416
	R	1 (0.29)	2 (0.8)		
Imipenem	S	331 (97.35)	251 (96.2)	0.677	0.411
	R	9 (2.65)	10 (3.8)		
Tetracycline	S	332 (96.51)	244 (93.5)	2.380	0.123
	R	13 (3.78)	17 (6.5)		
Cefixime	S	338 (99.41)	258 (98.9)	0.564	0.453
	R	2 (0.59)	3 (1.1)		
Ceftazidine	S	338 (99.71)	258 (98.9)	0.664	0.413
	R	1 (0.29)	2 (1.1)		
Cephalexin	S	331 (98.51)	250 (97.7)	0.583	0.445
	R	5 (1.29)	6 (2.3)		
Cotrimoxazole	S	263 (77.35)	227 (87.3)	9.752	0.**002***
	R	77 (22.65)	33 (12.7)		
Piperacillin	S	262 (78.68)	216 (86.4)	5.76	**0.016***
	R	71 (21.32)	34 (13.6)		
Aztreonam	S	324 (97.30)	251 (96.5)	0.286	0.593
	R	9 (2.70)	9 (3.5)		
Ampicilin	S	272 (80.47)	239 (91.9)	15.49	**<0.001****
	R	66 (19.53)	21 (8.1)		
Cefuroxime	S	333 (98.23)	251 (96.2)	2.415	0.120
	R	6 (1.77)	10 (3.8)		
Ciprofloxacin	S	49(14.50)	30 (11.5)	1.160	0.280
	R	289 (85.50)	231 (88.5)		
Gentamycin	S	3 (0.88)	1 (0.9)	0.557	0.456
	R	337 (99.12)	260 (99.1)		
Amikacin	S	2 (0.59)	4 (1.5)	1.315	0.252
	R	336 (99.41)	257 (98.5)		
Amoxyclav	S	325 (97.31)	255 (98.1)	0.378	0.539
	R	9 (2.69)	5 (1.9)		
Azithromycin	S	118 (34.71)	222 (85.5)	152.370	**<0.001****
	R	222 (65.29)	39 (14.9)		

Note: S - Sensitive, R - Resistant; *Statistically significant at LS=.05, **Highly statistically significant at LS=.001.

increased sensitivity of *S.* Typhi to imipenem. Two studies in Indonesia and China also noticed decreased resistance of *S.* Typhi to imipenem (Lugito and Cucunawangsih, 2017; Yaxian et al., 2015). However, we found alarmingly heightened resistance of *S.* Typhi against ciprofloxacin and azithromycin. Two relevant studies found a similar trend revealing excessive resistance of azithromycin and ciprofloxacin as 95.29 and 90.0%, respectively (Rahman, 2015; Vlieghe et al., 2012). Similarly, decreased ciprofloxacin susceptibility for *S.* Typhi has been witnessed by some studies in India recently (Chandel and Chaudhsry, 2001). In addition, a study in Pakistan reported the enhanced resistance of *S.* Typhi for ciprofloxacin, that is, consistent with our finding, but that same study found reduced sensitivity to ampicillin which is not consistent with our finding (Qamar et al., 2014). *S. typhi* was also highly resistant to antibiotics like gentamycin and amikacin. In sharp contrast to us, a community-based study in Indonesia showed almost no resistance against ceftriaxone or ciprofloxacin (Punjabi et al., 2013). The antibiotic resistance pattern may vary among the countries.

Furthermore, the current study revealed that *S. paratyphi A* was greatly sensitive to cefepime, ceftriaxone, imipenem, tetracycline, cefixime, ceftazidime, cephalexin, cotrimoxazole, piperacillin, aztreonam, amikacin, amoxiclav and cefuroxime. In agreement with this, *S.* Paratyphi *A* showed complete sensitivity to ceftriaxone (Bhatia et al., 2007). Interestingly, like *S.* Typhi strain, *S.* Paratyphi *A* also became resistant to ciprofloxacin. But, unlike *S.* Typhi which showed considerable insensitivity to azithromycin, *S.* Paratyphi *A* was sensitive against the same antibiotic. Earlier studies, contrarily to our outcomes, found azithromycin as highly sensitive to both *Salmonella* spp. (Chandey and Multani, 2012). We also observed a strikingly resemblance between *S.* Typhi and *S.* Paratyphi *A* as they both demonstrated similar enhanced insensitivity to two other antibiotics: gentamycin and amikacin. In contrast, Naheed et al. (2010) found that all *S.* Paratyphi *A* isolates were susceptible to all antimicrobial agents they tested. In Bangladesh, alarmingly, both *S.* Typhi and *S.* Paratyphi *A* lost the susceptibility to azithromycin.

Azithromycin's insusceptibility to both *S.* Typhi and *S.* Paratyphi *A* poses an emerging public health concern as treatment failures have been reported (Molloy et al., 2010). Over-use of ciprofloxacin and azithromycin resulting from over-the-counter availability and easy oral administration, coupled with incomplete dose treatment by them might contribute to their high antibiotic resistance in Bangladesh. In the present study, not any single antibiotic had complete susceptibility to the total *S. typhi* isolates tested. Unless this increasing antibiotic resistance rate for *Salmonella* is checked, options for treating enteric fever cases would be lost shortly. Bangladesh Government should cryingly implement a national guideline on the proper usage of antibiotics.

Conclusion

The study unraveled the current antibiotic resistance patterns of *S.* Typhi and *S.* Paratyphi *A* to help medical practitioners so that they can make informed decisions and provide better treatment for enteric fever patients. This study revealed male and children were more susceptible to enteric fevers. Both *S.* Typhi and *S.* Paratyphi *A* were equally highly resistant to ciprofloxacin, gentamicin, and amikacin. Several antimicrobials presented significant variation in resistance against *S.* Typhi and *S.* Paratyphi *A*. Researchers and policymakers could find this study helpful in prioritizing their research scopes to tackle the upcoming challenges of antibiotic resistance among enteric fever patients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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