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Original Article

Antimicrobial resistance, virulence genes and molecular subtypes of *S. Enteritidis* isolated from children in Shanghai

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Abstract

Introduction: Antimicrobial resistance of *Salmonella* poses a key threat to public health worldwide. *Salmonella* Enteritidis (*S.* Enteritidis) (119 isolates) from children under 10 years old with diarrhea in Shanghai from 2010-2012 were characterized for antimicrobial susceptibility, virulence gene profiles and pulsed field gel electrophoresis (PFGE) patterns.

Methodology: The minimum inhibitory concentration for the 119 *S.* Enteritidis isolates was determined using an agar dilution method. The presence of virulence genes was detected by polymerase chain reaction (PCR). All the isolates with antimicrobial resistance were subjected to PFGE analysis.

Results: Among these isolates, 71.4% (85) were resistant to sulfafurazole, 59.7% (71) were resistant to ampicillin, 47.1% (56) were resistant to streptomycin, 7.6% (9) were resistant to ceftiofur and 3.4% (4) were resistant to ceftriaxone. Multidrug resistance (MDR) was found in 63.9% (76) of the isolates; 23 resistance profiles were identified. All isolates harbored the *ssa*Q and *sop*E virulence genes in the 16 virulence profiles (VPs); VP1 accounted for 70.59% of the 119 isolates. There were 57 PFGE patterns among the 92 isolates tested, mainly grouped into five clusters (A to E). All of the 76 MDR isolates carried multiple virulence genes.

Conclusions: Our study provides useful microbiological data for the successful treatment of *S.* Enteritidis infections in Shanghai. Although broad spectrum antimicrobials may be useful in the treatment of invasive *S.* Enteritidis infections, clinicians need to be aware of common microbiological traits, because of the high prevalence of MDR.

Key words: *S.* Enteritidis; antimicrobial resistance; virulence gene; PFGE; childhood infections.

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Introduction

Salmonella enterica subspecies *enterica* is one of the most common foodborne pathogens [1], causing more than 93 million illnesses and 155,000 deaths worldwide, 85% of which were related to contaminated food [2,3]. *Salmonella enterica* serovars are recognized as a common cause of childhood infections all over the world, particularly gastroenteritis, bacteremia, and typhoid (enteric) fever [4]. Notably, the incidence rate among children under the age of five (69.5 infections per 100,000 children) was higher than the average incidence rate of *Salmonella enterica* infection, which was 17.6 illnesses per 100,000 people [5]. In 2001-2003, an outbreak of *Salmonella* (Newport, Poona and Sandiego) in children was reported in the United States, in which 70% of the infected patients were under 10 years old and 31% were infants under 1 year old [6]. A recent review indicates that *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) cause approximately 80% of Salmonellosis in children under 5 [7]. *S.* Enteritidis is a predominant serovar in both developing and developed countries [8] and the most common serovar in clinical cases among children [9]. Because children have relatively low immunity, they are susceptible to *S.* Enteritidis infection, especially children aged under 10 years. Therefore, children were chosen as subjects for this study of antibiotic resistance in *S.* Enteritidis in Shanghai.

Clinical treatment of severe salmonellosis is based on the prescription of antibiotics, including ampicillin, third and fourth generation cephalosporins and fluoroquinolones [10]. However, *Salmonella* isolates with multidrug resistance (MDR, defined as resistance

to three or more antimicrobials) have been found [11] and had increased to 70% by early in this century [12]. The spread of resistant *Salmonellae* is a particular concern for pediatricians because of the limited therapy options available for infants and children. The study of antimicrobial resistance in *Salmonellae*, especially in *S.* Enteritidis, has played an important role in public health research in the last two decades [13]. It is essential to continue to update information on antimicrobial treatments for children with invasive bacterial infections.

Shanghai is the economic and financial center of China and one of its largest cities, with more than 24 million inhabitants. The Shanghai Center for Disease Control and Prevention (SCDC) is one of eight sites in China supported by the WHO Global Foodborne Infectious Diseases Network (WHO GFN). However, the level of antimicrobial resistance in the most common serovar *S.* Enteritidis for children in a large metropolitan population like Shanghai was unknown. The incidence of these infections has been under-reported; updated data is needed to develop rational strategies for the clinical treatment of *S.* Enteritidis infection.

The objective of this study was to determine the antimicrobial susceptibility, virulence profile and genetic relatedness of *S.* Enteritidis isolates from children in Shanghai collected from 2010 to 2012.

Methodology

S. Enteritidis isolates

The *S.* Enteritidis isolates (n = 119) used in this study were collected from 11 hospitals located in eight major districts of Shanghai, (Jinshan, Baoshan, Changning, Xuhui, Jingan, Songjiang, Hongkou, Putuo), China. All isolates from local hospitals were obtained from children under the age of 10 showing diarrhea and infection, during the years from 2010-2012. API 20Etest strips (bioMerieux Vitek, Marcy-l'Étoile, France) were used for strain identification. All isolates were serotyped with commercial antiserum (S&A Reagents Laboratory, Bangkok, Thailand) and the serotypes were assigned according to the Kauffmann-White scheme [14]. The clinical isolates were stored in 25% glycerol at -80°C until use.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) were determined using an agar dilution method according to the guidelines recommended by the Clinical and Laboratory Standard Institute [15]. The following antimicrobials were tested: amikacin (AMK), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), ampicillin (AMP), ceftiofur (EFT), ceftriaxone (CRO), sulfisoxazole (SUL), chloramphenicol (CHL), ciprofloxacin (CIP), ofloxacin (OFX), tetracycline (TET), doxycycline (DOX). *Escherichia coli* strain ATCC 25922 was used for quality control. Breakpoints were based on CLSI standards (2010) [16] except for

Table 1. Primer of virulence genes in this study.

Genes	Location	Function	Primers	PCR product size (bp)
avrA	SPI 1	Inhibits the key proinflammatory, antiapoptotic NF-kappa B pathway	GTTATGGACGGAACGACATCGG ATTCTGCTTCCCGCCGCC	385
ssaQ	SPI 2	Secretion system apparatus protein, component of second T3SS	GAATAGCGAATGAAGAGCGTCC CATCGTGTTATCCTCTGTCAGC	677
mgtC	SPI 3	Intramacrophage survival protein	TGACTATCAATGCTCCAGTGAAT ATTTACTGGCCGCTATGCTGTTG	655
siiD	SPI 4	Hemolysin D family secretion protein	GAATAGAAGACAAAGCGATCATC GCTTTGTCCACGCCTTTCATC	1231
sopB	SPI 5	Translocated effector protein (phosphoinositide phosphatase) via T3SS	GATGTGATTAATGAAGAAATGCC GCAAACCATAAAAACTACACTCA	1170
gipA	Gifsy-1 bacteriophage	Peyer's patch-specific virulence factor	GCAAGCTGTACATGGCAAAG GGTATCGGTGACGAACAAAT	212
sodC1	Gifsy-2 bacteriophage	Periplasmic copper/zinc-superoxide dismutases	CCAGTGGAGCAGGTTTATCG GGTGCGCTCATCAGTTGTTC	460
sopE	SopEPhi bacteriophage	Translocated T3SS effector protein	ACACACTTTCACCGAGGAAGCG GGATGCCTTCTGATGTTGACTGG	398
spvC	pSLT plasmid	Hydrophilic protein, provides rapid growth and survival within host	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	571
bcfC	Chromosome	Bovine colonization factor, fimbrial usher	ACCAGAGACATTGCCTTCC TTCTGATCGCCGCTATTCG	467

streptomycin (64 μg/mL), which was based on a previous study [17].

PCR detection of virulence genes

DNA was extracted from the *S.* Enteritidis isolates using a bacteria genomic DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions. DNA concentration and purification was measured using Nanodrop 2000c. The DNA was tested for ten virulence genes by PCR (Table 1) [18-23]. PCR reactions were carried out in a volume of 25 μL containing 12.5 μL 2×Taq Plus Master Mix (Vazyme Biotech, Nanjing, China), 9 μL ddH_2O, 1 μL of forward and reverse primers and 1.5 μL DNA template. Negative controls using dH_2O instead of template DNA were included in all PCR reaction sets. PCR products were analyzed using electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under UV light.

Pulsed-field gel electrophoresis (PFGE)

According to the protocol developed by the Centers for Disease Control and Prevention (CDC) for Molecular Subtyping of *Salmonellae*, all resistant isolates (92) were subjected to PFGE analysis (PulseNet, CDC, Atlanta, GA, USA) (CDC 2012). Briefly, agarose-embedded DNA was digested with 50 U of restriction enzyme *Xba* I (TaKaRa, Dalian, China) for 1.5-2 hours at 37°C. The restriction fragments were separated by electrophoresis in 0.5 × TBE buffer at 14°C for 18-19 hours using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA, USA) with the pulse times set to 2.16 - 63.8 sec. *Salmonella* Braenderup H9812 was used as a molecular reference

Table 2. Resistance patterns of the 119 *S.* Enteritidis isolates.

Resistance pattern	% Prevalence (no.)
AMP-CRO-EFT-KAN-STR-TET-DOX-OFX-SUL	0.8% (1)
AMP-GEN-KAN-TET-DOX-CIP-OFX-SUL	0.8% (1)
AMP-CRO-STR-TET-DOX-OFX-SUL	0.8% (1)
AMP-EFT-GEN-STR-TET-DOX-SUL	0.8% (1)
AMP-GEN-KAN-TET-CIP-OFX-SUL	1.7% (2)
EFT-GEN-KAN-STR-TET-DOX-SUL	0.8% (1)
AMP-EFT-STR-TET-DOX-SUL	0.8% (1)
AMP-GEN-KAN-TET-DOX-SUL	0.8% (1)
AMP-GEN-TET-CIP-OFX-SUL	0.8% (1)
AMP-CRO-EFT-STR-SUL	0.8% (1)
AMP-GEN-TET-DOX-SUL	0.8% (1)
AMP-STR-TET-DOX-SUL	11.8% (14)
AMP-CRO-STR-SUL	0.8% (1)
AMP-GEN-KAN-SUL	6.7% (8)
STR-TET-DOX-SUL	1.7% (2)
EFT-GEN-KAN-SUL	0.8% (1)
AMP-GEN-KAN	1.7% (2)
EFT-GEN-SUL	0.8% (1)
AMP-GEN-SUL	0.8% (1)
AMP-STR-SUL	19.3% (23)
TET-DOX-SUL	5.9% (7)
AMP-TET-STR	0.8% (1)
TET-DOX-STR	0.84%(1)
STR-TET-SUL	1.7% (2)
AMP-GEN	0.8% (1)
AMP-STR	2.5% (3)
STR-SUL	0.8% (1)
STR-TET	0.8% (1)
TET-DOX	0.8% (1)
EFT-SUL	0.8% (1)
TET-SUL	0.8% (1)
AMP-SUL	0.8% (1)
AMP-EFT	0.84% (1)
AMP	3.7% (4)
Pan-susceptible	24.4% (27)

marker. The DNA bands were visualized under UV light. An unweighted pair group method with arithmetic averages (UPGMA) was employed, using BioNumerics 7.6 (Applied Maths, Saint-Martens-Latem, Belgium) to generate dendrograms for pattern analysis. The Dice coefficient and tolerance was set to 1.5%.

Results

Among the 119 clinical isolates of *S*. Enteritidis, 27 (24.4%) were susceptible to all 13 antimicrobials (Table 2). The highest percentage of resistance was detected to sulfisoxazole (71.4%), followed by ampicillin (55.7%), streptomycin (47.1%), tetracycline (35.3%), doxycycline (27.7%), gentamicin (18.5%), kanamycin (14.3%), ceftiofur (7.6%), ofloxacin (5.0%) and ceftriaxone (3.4%). No isolate was resistant to amikacin, ciprofloxacin or chloramphenicol (Table 3).

Ninety-two (77.3%) of the 119 isolates exhibited resistance to at least one antimicrobial and 76 (63.9%) were MDR (Table 2). The percentage of isolates resistant to the third-generation cephalosporins (ceftriaxone (3.4%) and ceftiofur (7.6%)) and fluoroquinolone antibiotics (ciprofloxacin (0) and ofloxacin (5.0%)) was lower than the percentage of isolates resistant to aminoglycosides (streptomycin (47.1%), gentamicin (18.5%), kanamycin (14.3%)), penicillins (ampicillin (55.7%)) and sulfisoxazole (71.4%). Most isolates had low MICs to the third-generation cephalosporins (ceftriaxone and ceftiofur) and fluoroquinolone antibiotics (ciprofloxacin and ofloxacin); the MIC50 was equal to or less than 1 μg/ml, while the MIC90 were both less than 1 μg/mL (Table 3).

Twenty-three MDR profiles were detected among 92 resistant isolates, including one that showed resistance to 9 out of the 13 antimicrobials on the panel (AMP-CRO-EFT-KAN-STR-TET-DOX-OFX-SUL). Among the rest of the MDR profiles, the dominant resistant profiles were AMP-STR-SUL (n = 23), AMP-STR-TET-DOX-SUL (n = 14), AMP-GEN-KAN-SUL (n = 8) and TET-DOX-SUL (n = 7). Other profiles were found sporadically with the majority found in only one isolate (25 profiles). (Table 2).

The presence of virulence genes *ssa*Q and *sop*E was detected in all isolates. The incidence of other virulence genes, *avr*A, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *spv*C and *bcf*C, were 98.3%, 95.0%, 96.6%, 97.5%, 98.3%, 92.4%, 88.2% and 95.8%, respectively. Seventy-five isolates harbored all ten virulence genes, while otherswere positive for at least eight genes (Figure 1). We identified 16 different virulence profiles among the 119 *S*. Enteritidis isolates. The dominant profile was virulence profile 1 (VP1, 70.6%) which contained all of the genes, followed by VP2 (5.9%), VP3 (4.2%) and VP4 (3.4%). Of the remaining profiles, two profiles consisted of three isolates, three profiles consisted of two isolates and the other seven profiles were represented by a single isolate each (Table 4).

A total of 57 PFGE patterns were observed in 92 isolates with a similarity of position of DNA fragments ranging between 47.0% and 100%. Seventy-six PFGE profiles were grouped into five clusters (A to E). The predominant cluster was cluster C, which contained 27 isolate, 19 of which had the same pattern. Isolates from children in different age groups were represented in the

Table 3. Distribution of MIC among 119 *S.* Enteritidis isolates.

Antimicrobial agent	Distribution (no.) of MIC (μg/mL) among the 119 isolates																
	≤ 0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512	MIC50	MIC90	Resistant breakpoint	Resistance% (no.)
Ampicillin					9	39					71			128	128	≥32	59.7% (71)
Ceftriaxone	115							4						≤ 0.125	≤ 0.125	≥ 4	3.7% (4)
Ceftiofur			10	79	19	2		1	8					1	2	≥ 8	7.6% (9)
Amikacin		9		105	5									1	1	≥ 64	0
Gentamicin	2		25	69	1			1		21				1	64	≥ 16	18.5% (22)
Kanamycin				66	23	10		3		3	12	2		1	128	≥ 64	14.3% (17)
Streptomycin					19	2	4	4	35		36	69		32	256	≥ 64	47.1% (56)
Tetracycline				35	35	4	3	19		23				2	64	≥ 16	35.3% (42)
Doxitard				2	76	6	2	12	5	16				2	64	≥ 16	27.7% (33)
Ciprofloxacin	107	5	3	2	2									≤ 0.125	≤ 0.125	≥ 1	0
Ofloxacin	6	11	80	16	5	1								0.5	1	≥ 2	5.0% (6)
Chloramphenicol					104	13	1	1						2	4	≥ 32	0
Sulfisoxazole								1	2	8	12	19	85	≥ 512	≥ 512	≥ 512	71.4% (85)

The number in the table represent the number of isolates at each dilution level. Blanks within the table indicate the number to be zero. Resistance % represents the proportion of resistant isolates (number). MIC50: The minimum inhibitory concentration that inhibits the growth of 50% of the isolates. MIC90: The minimum inhibitory concentration that inhibits the growth of 90% of the isolate.

same patterns; there was no correlation between the *S.* Enteritidis PFGE profile and patient age.

Discussion

We found high levels of antimicrobial resistance to sulfisoxazole (71.4%), ampicillin (59.7%) and streptomycin (47.1%) in *S. Enteritidis* isolated. These results were similar to findings from Beijing, China on clinical samples of children from 2010 to 2014 showed

Figure 1. *Xba*IPFGE patterns, antimicrobial resistance profiles and virulence gene profiles of isolates in this study. A black box indicates resistance to a particular antimicrobial or positive for a particular virulence gene.

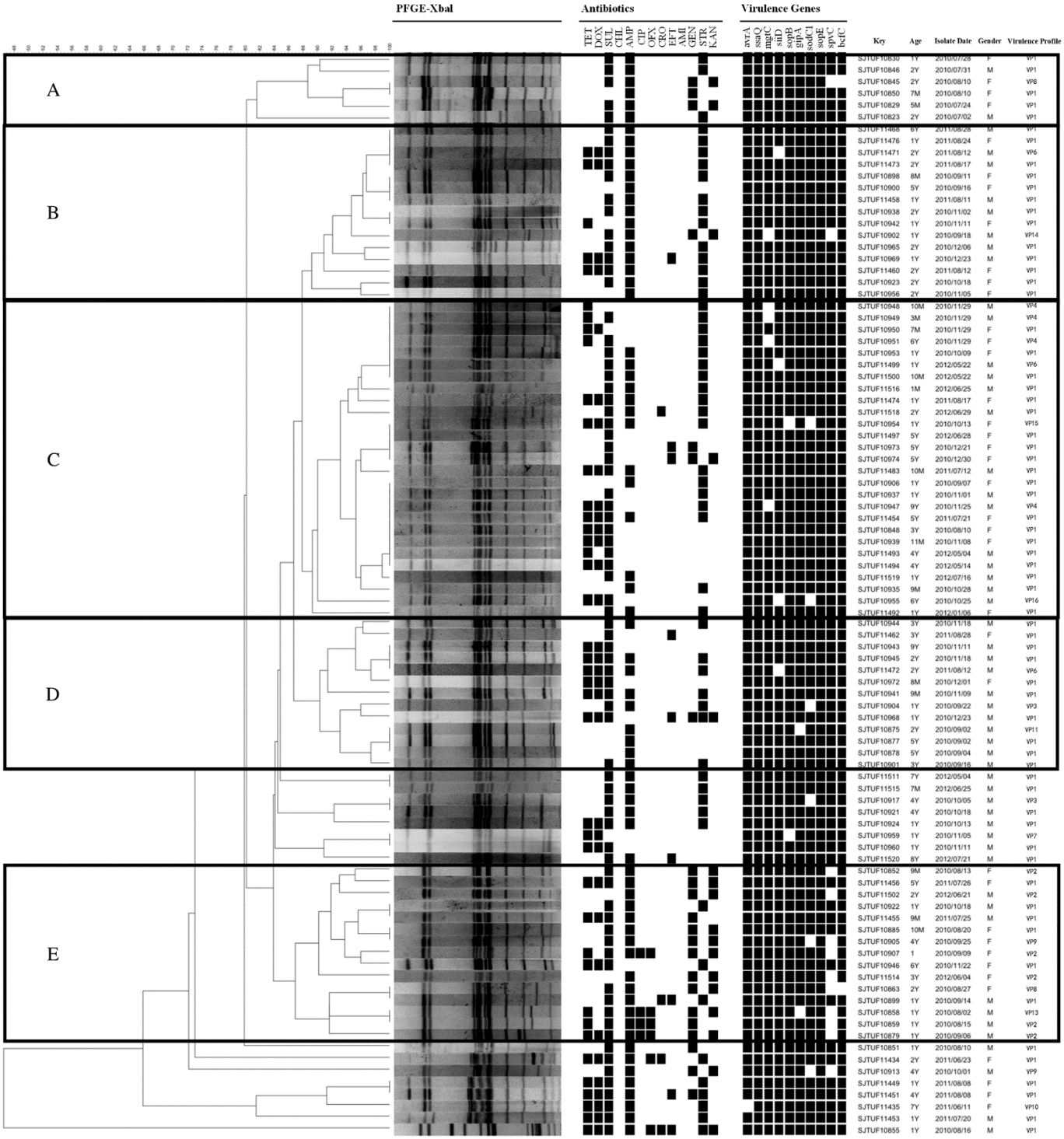

Table 4. Virulence gene profiles among the 119 *S.* Enteritidis strains.

Virulence profile	% prevalence (no.)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C-*spv*C(VP1)	70.6% (84)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C(VP2)	5.9% (7)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*bcf*C-*spv*C(VP3)	4.2% (5)
*ssa*Q-*sop*E-*avr*A-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C-*spv*C(VP4)	3.4% (4)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*sod*C1-*spv*C(VP5)	2.5% (3)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sop*B-*gip*A-*sod*C1-*bcf*C-*spv*C(VP6)	2.5% (3)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*gip*A-*sod*C1-*bcf*C-*spv*C(VP7)	1.7% (2)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*sod*C1 (VP8)	1.7% (2)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*gip*A-*bcf*C(VP9)	1.7% (2)
*ssa*Q-*sop*E-*mgt*C-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C-*spv*C(VP10)	0.8% (1)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*sod*C1-*bcf*C-*spv*C(VP11)	0.8% (1)
*ssa*Q-*sop*E-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C-*spv*C(VP12)	0.8% (1)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*sop*B-*sod*C1-*bcf*C(VP13)	0.8% (1)
*ssa*Q-*sop*E-*avr*A-*sii*D-*sop*B-*gip*A-*sod*C1-*bcf*C(VP14)	0.8% (1)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sii*D-*gip*A-*bcf*C-*spv*C(VP15)	0.8% (1)
*ssa*Q-*sop*E-*avr*A-*mgt*C-*sop*B-*gip*A-*bcf*C-*spv*C(VP16)	0.8% (1)

resistance to sulfisoxazole in 54.3% of the isolates, resistance to ampicillin in 60.0% and resistance to streptomycin in 42.9% [24]. Indeed, the antimicrobial resistance rates of sulfisoxazole, ampicillin and streptomycin were even higher in our study, which may indicate that the antimicrobial resistance rates had increased during 2010, 2011 and 2012. Approximately 63.9 % of the isolates were resistant to at least three antimicrobials. The predominant resistance pattern (AMP-STR-SUL) was similar to that in the previous study in Beijing [24]. Despite the similarity in the pattern of resistance, the frequency was higher in our study and the trend of multidrug resistance was more obvious. It is worth mentioning that one of the 119 isolates in our study exhibited resistance to nine antimicrobial agents. The high level of MDR found in our research serves as a warning for other major cities in China [24,25] and should be considered when reviewing appropriate treatment options.

The prevalences of the virulence genes *sop*B and *spv*C were 97.48% and 88.24% in our study, almost identical to a previous report on poultry isolates [26]. The plasmid-associated gene *spv*C is often detected in *S.* Enteritidis and a few other *Salmonella* serovars; it was found in more than 80% of the isolates we tested[27]. The *avr*A, *ssa*Q, *mgt*C, *sii*D, and *sop*B genes were highly conserved and were genetic markers for the presence of the SPI 1-5 in *Salmonella* [28]. The high percentage of these virulence genes in our study also indicated that they were widespread and highly conserved. Information on the distribution of virulence genes can also reflect and improve the understanding of potential risks for human infections.

PFGE genotyping can be used to ascertain the homology of the same serotype isolates and determine whether they were derived from an outbreak or not. PFGE analysis in our study indicated that the *S.* Enteritidis isolates did not cluster by age, isolation date or gender, as isolates from different ages clustered with the same PFGE pattern. For example, in cluster C, eight isolates recovered from children who were 11 months to 9 years of age shared an identical pattern. These isolates were recovered between 2010 and2012, suggesting that the general characteristics of the *Salmonella* population did not change substantially during these years. PFGE typing of *S.* Enteritidis also showed a relatively high diversity indicating that the cases were sporadic and not part of any major outbreak. In our study, the same PFGE pattern was found in different *Salmonella* isolates from different months. This indicates this particular strain is successful in its current niche over a longer period of time, in comparison to the sporadic strains, and could pose a risk of an epidemic outbreak of *Salmonella* infection in the future. As mentioned, *S.* Enteritidis strains had a high prevalence in poultry products [29]. It was very likely that children have been exposed to such animals or have consumed poultry-related foods in these urban areas. Hence, supervision of children's diet, such as ensuring that chicken is properly cooked, should be strengthened. Meanwhile, children should wash their hands frequently to reduce the possibility of infection.

All of our VP4 profiles were grouped into cluster C and most of VP2 were grouped into cluster E. The dominating virulence profiles were distributed among clusters, which was contrary to results using the serotype grouping method, which would group the

dominant virulence profiles in the same cluster [30]. In previous studies [31], *Salmonella* isolates with the same PFGE patterns were more likely to have similar antibiotic resistance profiles. This was not observed in our study.

Conclusions

Our study provided data on the distribution of antimicrobial resistance in *S.* Enteritidis isolates from children with clinical disease in Shanghai from 2010-2012. Most isolates (85) had some antimicrobial resistance and some (76) were MDR. Furthermore, isolates taken from children of different ages on different isolation dates had the same PFGE patterns, indicating these could be linked. These results highlight the need for continuous surveillance and monitoring of potential epidemic outbreaks of MDR *S.* Enteritidis in China. They also provide a useful view of antimicrobial resistance patterns needed by pediatricians to decide on treatment options. If antimicrobial treatment is necessary, the third-generation cephalosporins (ceftriaxone and ceftiofur) can still be considered for the treatment of *S.* Enteritidis infections in children.

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Authors' contributions

Li Xu, Xiujuan Zhou and Xianming Shi designed the experiments, Li Xu and Xiujuan Zhou performed the experiments. Li Xu and Xiujuan Zhou wrote the paper. Xuebin Xu provided isolates. Yue Liu, Dai Kuang and Karl R. Matthews revised the paper. All the authors read and approved the final manuscript.

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