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ORIGINAL ARTICLE

Antimicrobial Resistance, Genetic Diversity and Virulence Genes of Salmonella Typhimurium Isolated in Infant with Acute Diarrhea in Fuzhou, China, 2015 - 2017

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SUMMARY

Background: **S. Typhimurium was the dominant serovar in an infant in Fuzhou, China. There have been few comprehensive studies on Salmonella typhimurium in infants in China.**
Methods: **We conducted a retrospective study on 30 Salmonella typhimurium from 3,200 fecal samples of infants with acute diarrhea from 2015 to 2017. Thirty S. Typhimurium strains were tested for antimicrobial susceptibility and characterized for virulence genes. Pulsed-field gel electrophoresis (PFGE) was also applied for comparison of genetic relatedness.**
Results: **All of the strains harbored misL, orfL, pipD, prgH, sifA, sopB, sitC, spiC, and invA genes. The other three gene distributions in the strains are different. Strains subtyped into 4 virulotypes (VP1-VP4), the most common virulence profile was VP3, accounting for 63.3% of the strains. The resistance to ciprofloxacin and ceftriaxone was 26.7%. The proportion of MDR isolates is approximately 90.0%. Sixteen different antimicrobial resistance patterns were observed and the most frequent resistance type was antibiotype 13 (resistance to streptomycin, tetracycline, amoxicillin), occurring in 43.3% of the isolates. Regarding PFGE, 30 isolates of S. Typhimurium showed genetic diversity, while no predominant PFGE patterns were observed in S. Typhimurium. Moreover, no correlation between virulence profiles or antibiotic patterns and PFGE clusters was observed. With one exception, VP1 which harbors pefA showed more diversity than the other virulence profiles among PFGE profiles.**
Conclusions: **Our study provided valuable information on virulence gene content, antibiotic resistance, and genetic diversity of S. Typhimurium isolated from infant with acute diarrhea in Fuzhou, China.**
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KEY WORDS

Salmonella typhimurium, infants, antimicrobial resistance, genetic diversity, virulence genes

INTRODUCTION

WHO indicated that diarrheal infections caused by Salmonella species was one of the greatest burden of foodborne disease globally [1,2]. Salmonella spp. was an important reason of diarrheal illness in infants among which S. Typhimurium is one of the dominant serovars in many countries [3,4]. Similarly, during the period from 2014 to 2015, the majority of the Salmonella isolates in hospitals across China was S. Typhimurium and S. Enteritidis [5]. After 2008, the number and proportion of S. Typhimurium rose significantly, exceeding Salmonella Enteritidis and becoming the dominant serovar in many regions of China [6,7]. Ke B found that S. Typhimurium affected infants, whereas S. Enteritidis affected adults. The eating habits of infants and adults or special host adaptations may explain the observed distribution pattern [7]. Meanwhile, a worrying percentage of multidrug-resistant (MDR) strains, including those resistant to cephalosporin and quinolones were also observed in S. Typhimurium in many countries [8-10]. These antibiotics always are the priority choice for the treatment of invasive salmonellosis in humans. S. Typhimurium pathogenicity was in connection with many virulence factors which help the pathogen in adhesion, invasion, intramacrophage survival, antibiotic resistance, systemic infection, fimbrial production, toxin production, and Mg2+ and iron transport [11,12]. Most virus-related genes are located on a virulence-associated plasmid (pSTV) and chromosomal Salmonella Pathogenicity Islands (SPIs). At least 17 SPIs have been identified in different Salmonella serovars [13]. SPI-1 and SPI-2, which are associated with type 3 secretion systems (T3SS), are the most prominent SPIs. The SPI-1 encoded genes such as invA, sopB, sopE, and prgH allow S. Typhimurium to invade phagocytic and non-phagocytic cells, while the SPI-2 encoded genes such as spiC and sifA enable S. Typhimurium to survive and replicate in host cells, macrophages in particular [13, 14]. Meanwhile, a few parts of S. Typhimurium harbor virulence plasmids which encode genes related to systemic disease [15].

Pulsed-field gel electrophoresis (PFGE) is a widely used method in microbial epidemiology [16,17]. The international molecular subtyping network (PulseNet) contributing for surveillance of foodborne disease, regards PFGE as the gold standard for molecular typing of Salmonella to understand microbial epidemiology [17,18]. There have been few comprehensive studies on S. Typhimurium from infants in Fuzhou, China. In this study, the objectives were to investigate frequencies of virulence genes and antibiotic resistance in S. Typhimurium strains isolated from infants with diarrhea between 2015 and 2017. Furthermore, we utilized PFGE to determine the clone relatedness and to illustrate any epidemiological relationship between these strains.

MATERIALS AND METHODS

Sample collection

A total of 30 non-duplicate S. Typhimurium clinical isolates were consecutively collected from infants with diarrhea and other clinical symptoms such as fever, vomiting or abdominal pain at a Chinese 24 sentinel hospital (The Fujian Provincial Maternity and Infant's Hospital, affiliated hospital of Fujian Medical University, Fuzhou, China) between 2015 and 2017. Clinical demographic information of each case was also collected, including sample number, name, gender, age, symptoms, date of disease onset and date of sample collection. Isolates were confirmed as Salmonella by VITEK gram-negative identification cards (bioMerieux Inc., Hazelwood, MO, USA) and were serotyped with Salmonella diagnostic serum (Ningbo Tianrunbio-Pharmaceutical Co., Ltd). The isolates were stored in Baso preservative tubes (Baso Diagnostics, Inc., Zhuhai) at -80°C until needed.

Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed by disk diffusion test containing thirteen antimicrobials, ciprofloxacin (5 μg), nalidixic acid (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), streptomycin (10 μg), tetracycline (30 μg), amoxicillin (20 μg), meropenem (10 μg), chloramphenicol (30 μg), and piperacillin-tazobactam (100/10 μg). All disks were obtained from Oxide, Ltd. (Cambridge, United Kingdom). The results were interpreted according to the breakpoints of the CLSI guidelines [19]. Escherichia coli ATCC25922 was used as routine quality control. MDR was defined as resistance to more than three types of antimicrobials.

Detection of virulence genes

Genomic DNA of the S. Typhimurium isolates was extracted by boiling method. The presence of 12 known virulence genes, which were related to pathogenicity, were screened by polymerase chain reaction (PCR) [12, 20]. Virulence profiles were observed among the strains according to the combinations of virulence genes (Table 1).

Pulsed-Field Gel Electrophoresis (PFGE)

In order to understand the genetic relatedness of S. Typhimurium isolates, isolates were analyzed by PFGE using XbaI (New England Biolabs, Leusden, The Netherlands) digestion as previously described [21]. A PFGE dendrogram was constructed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) according to the UPGMA (unweighted pair group method) using Dice similarity coefficients.

Table 1. Primers used in this study.

Gene	Nucleotide Sequences (5' → 3')	Amplicon size (bp)	Reference
prgH	F: GCCCGAGCAGCCTGAGAAGTTAGAAA	756	[12]
	R: TGAAATGAGCGCCCCTTGAGCCAGTC		
sopB	F: CGGACCGCCCAGCAACAAAACAAGAAGAAG	220	[12]
	R: TAGTGATGCCCGTTATGCGTCAGTGTATT		
sopE	F: TCAGTTGGAATTGCTGTGGA	642	[20]
	R: TCCAAAAACAGGAAACCACAC		
invA	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT	1070	[12]
	R: AGTTTCTCCCCCTCTTCATGCGTTACCC		
sitC	F: CAGTATATGCTCAACGCGATGTGGGTCTCC	768	[12]
	R: CGGGGCGAAAATAAAGGCTGTGATGAAC		
spiC	F: CCTGGATAATGACTATTGAT	301	[20]
	R: AGTTTATGGTGATTGCGTAT		
sifA	F: TTTGCCGAACGCGCCCCCACACG	449	[12]
	R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT		
misL	F: GTCGGCGAATGCCGCGAATA	561	[20]
	R: GCGCTGTTAACGCTAATAGT		
orfL	F: GGAGTATCGATAAAGATGTT	332	[20]
	R: GCGCGTAACGTCAGAATCAA		
pipD	F: CGGCGATTCATGACTTTGAT	399	[20]
	R: CGTTATCATTCGGATCGTAA		
iroN	F: ACTGGCACGGCTCGCTGTCGCTCTAT	1205	[12]
	R: CGCTTTACCGCCGTTCTGCCACTGC		
pefA	F: GCGCCGCTCAGCCGAACCAG	157	[12]
	R: GCAGCAGAAGCCCAGGAAACAGTG		

RESULTS

Clinical distribution characteristics

Between January 2015 and December 2017, a total of 3,200 stool specimens were cultured from infants with diarrhea in Fuzhou, resulting in 69 (2.16%) Salmonella isolates. The predominant serotype S. Typhimurium (43.48%, 30/69) constituted nearly half of all the isolates. Among 30 S. Typhimurium, most of the Salmonella isolates were found in infants < 2 years of age, accounting for 96.66% (29/30) and the > 2 year-age-group accounting for only 3.33% (1/29). The male-to-female ratio was 1.14:1. Such infections usually are more frequent in summer and autumn (May - October) accounting for 60.0%.

Antimicrobial resistance situation and resistance profiles

In total, 30 S. Typhimurium isolates were isolated during the study period, 27 of 30 (90.0%) isolates were resistant to three or more antimicrobials (MDR). S. Typhimurium isolates were highly resistant to tetracycline (96.7%), amoxicillin (93.3%), and streptomycin (86.6%). Of note, resistance to the third generation cephalosporins such as ceftriaxone and ceftazidime was up to 26.7% and 16.6%, respectively, while resistance rates to fluoroquinolone like nalidixic acid and ciprofloxacin was 26.7%. However, 36.7% of all isolates (n = 30), showed a reduced susceptibility to ciprofloxacin (n = 11). All of the isolates were susceptible to meropenem and only one isolate was resistant to piperacillin/tazobactam (Figure 1). The isolates showed 16 different antimicrobial resistance patterns and the most prevalent resistance type was antibiotype 13 (resistance to streptomycin, tetracycline, and amoxicillin), accounting for 43.3% of the isolates (Table 2).

Distribution of virulence genes and virulence gene profiles

As for virulence genes, all of the S. Typhimurium strains harbored misL, orfL, pipD, prgH, sifA, sopB, sitC, spiC, and invA, with iroN genes having a preval-

Table 2. Profile of antibiotic resistance in S. Typhimurium isolates.

Antibiotypes	Resistance pattern	No. (%)
Ab1	CIP-NA -CTX-CAZ-CRO-FEP -S -T-SXT-A- CPM	1 (3.33)
Ab2	CIP-NA -CTX-CRO -FEP -S -T-SXT-A -CPM	1 (3.33)
Ab3	CIP -CTX-CAZ-CRO- S -T- A- CPM-TZP	1 (3.33)
Ab4	NA -CTX- CRO-FEP- S -T- A- CPM	1 (3.33)
Ab5	NA -CTX- CRO- S -T-SXT-A -CPM	1 (3.33)
Ab6	CIP-NA - S-T-SXT-A -CPM	2 (6.67)
Ab7	CTX-CAZ-S-FEP-CRO-T-A	1 (3.33)
Ab8	CTX-CAZ-S-CRO-T-A	2 (6.67)
Ab9	CIP-NA- S - T -A -CPM	1 (3.33)
Ab10	CIP-NA -T-SXT-A -CPM	1 (3.33)
Ab11	CIP-NA -T-A -CPM	1 (3.33)
Ab12	T-SXT-A -CPM	1 (3.33)
Ab13	S - T -A	13 (43.3)
Ab14	S - T	1 (3.33)
Ab15	S - A	1 (3.33)
Ab16	T	1 (3.33)

Abbreviations: CTX - Cefotaxime, CAZ - Ceftazidime, S - Streptomycin, FEP - cefepime, CRO - ceftriaxone, T - Tetracycline, SXT - trimethoprim/sulfamethoxazole, A - amoxicillin, MEM - meropenem, CPM - Chloramphenicol, CIP - Ciprofloxacin, TZP - piperacillin/tazobactam, NA - Nalidixic acid.

Table 3. Distribution of virulence genes.

Virulence genes	No. (%)
prgH	30 (100)
sopB	30 (100)
sopE	7 (23.3)
sitC	30 (100)
spiC	30 (100)
sifA	30 (100)
misL	30 (100)
orfL	30 (100)
pipD	30 (100)
iroN	26 (86.7)
pefA	1 (3.3)
invA	30 (100)

ence rate of 86.7%. The gene with the lower prevalence rates was sopE (23.3%). Meanwhile, the plasmid encoded fimbriae (pefA) gene was found to be present in 3.3% (1/30) of the isolates (Table 3). S. Typhimurium strains could be subtyped into 4 virulotypes (VP1 - VP4) based on the presence and absence of these 12 virulence genes (Table 1). The most common virulence profile was VP3 which accounted for 63.3% (n = 19) of the strains, followed by VP2 (20.0%), whereas VP1 was the least prevalent one and only in 1 strain (Table 4).

PFGE profiles

Regarding PFGE, 30 isolates of S. Typhimurium showed genetic diversity, while no predominant PFGE pat-

Table 4. Virulence profiles (virulotypes) of S. Typhimurium isolates.

Virulence profile	Virulence genes												Isolates No. (%)
	misL	orfL	pipD	prgH	sifA	sopB	sitC	spiC	invA	iroN	sopE	pefA	
VP1	+	+	+	+	+	+	+	+	+	+	+	+	1 (3.3)
VP2	+	+	+	+	+	+	+	+	+	+	+	-	6 (20.0)
VP3	+	+	+	+	+	+	+	+	+	+	-	-	19 (63. 3)
VP4	+	+	+	+	+	+	+	+	+	-	-	-	4 (13.3)

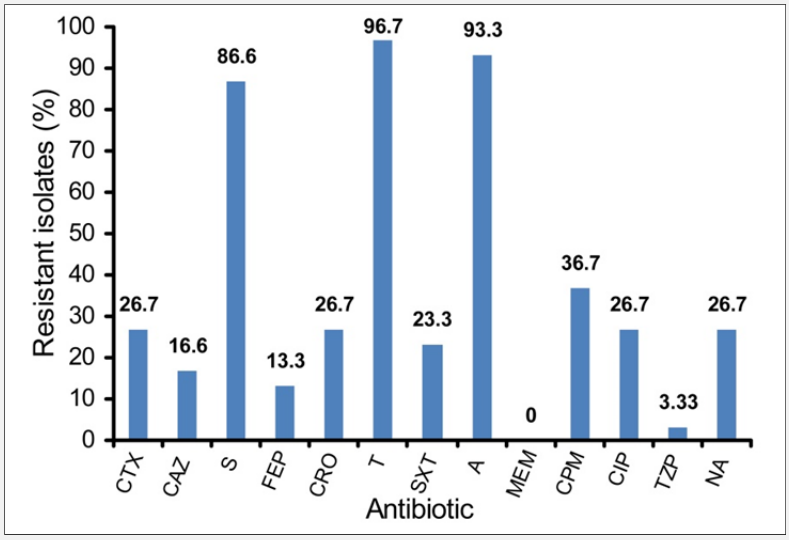

Figure 1. Antimicrobial resistances of 30 S. Typhimurium isolates.

CTX - Cefotaxime, CAZ - Ceftazidime, S - Streptomycin, FEP - cefepime, CRO - ceftriaxone, T - Tetracycline, SXT - trimethoprim/sulfamethoxazole, A - amoxicillin, MEM - meropenem, CPM - Chloramphenicol, CIP - Ciprofloxacin, TZP - piperacillin/tazobactam, NA - Nalidixic acid.

terns were observed in S. Typhimurium. There was almost no association between antibiotypes or virulence profiles and PFGE clusters. With one exception, virulence profile VP1, which harbors pefA, showed more diversity than the other virulence profiles among PFGE profiles (Figure 2).

DISCUSSION

Salmonella spp is one of the leading causes of foodborne diseases worldwide, accounting for a great part of outbreaks and sporadic cases in humans [22]. So far, there are more than 2,500 Salmonella serotypes have been identified, but the pathogenicity of each serotype leading to human illness is known to vary greatly [23]. In the US, CDC surveillance data revealed that S. Enteritidis was the most common serotype in clinical isolates, followed by Salmonella Newport and S. Typhimurium [3]. In contrast, in China S. Typhimurium seems to be more prevalent than S. Enteritidis [5], similar to Iraq [4]. Particularly after 2008, the number and proportion of S. Typhimurium rose significantly, surpassing S. Enteritidis and becoming the leading serovar [6-7]. However, we still did not know what caused this shifting from S. Enteritidis to S. Typhimurium; perhaps various efforts to control the spread of the pathogens through eggs, a main source of S. Enteritidis infections [24], caused the shift. Ke B found that S. Typhimurium affected infants, whereas S. Enteritidis affected adults [7].

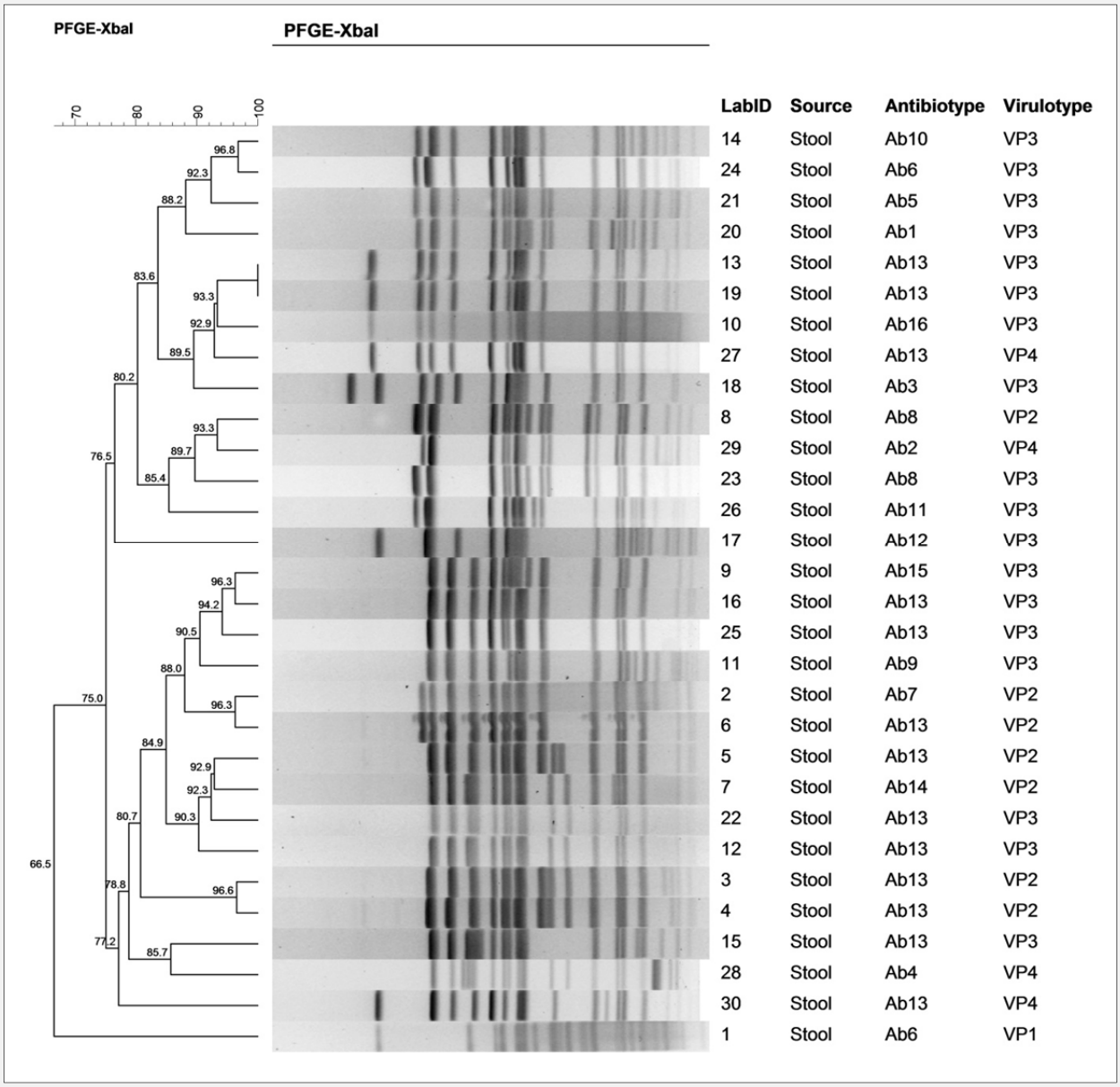

Figure 2. Dendrogram of the PFGE patterns of chromosomal DNA restriction fragments from 30 S. Typhimurium.

The dendrogram was constructed using the UPGMA method. Dice coefficients (percentages) are listed in the scale on the top of the dendrogram. Strain number, sample source, antibiotype, and virulotype are included.

The infant and adult dietary habits or special host immunity may explain this pattern of distribution. In our study, S. Typhimurium (44.93%) were the dominant serovar with infants in Fuzhou. Our hospital is a 3A (Class Three/Grade A) Women 's and Infant's Hospital in China. Our research object is infants. So, our data showed that S. Typhimurium is the dominant serovar whose proportion is significantly exceeding the other serovars in infants. Most of the S. Typhimurium isolates were found in infants < 2 years of age, accounting for 96.66% (29/30). Not only that, S. Typhimurium's resistance rate is higher than the other serovars. High drug-resistance in medicine has become a global focus [25]. Therefore, we should pay close attention to S. Typhimurium. There have been few comprehensive studies on S. Typhimurium in China, particularly with infants.

These studies illustrated antimicrobial resistance, genetic diversity, and virulence genes of S. Typhimurium isolated from infants with diarrhea.

Our findings demonstrated high level of ampicillin resistance (93.3%) among S. Typhimurium isolates. According to these results, it can be stated that ampicillin has no applicable role in the treatment of S. Typhimurium in our region. For this situation, fluoroquinolones are not approved by the FDA in people younger than 18 years of age and are not recommended unless the benefits of therapy outweigh the potential risks with use of the drug [26]. Therefore, third-generation cephalosporins are considered alternative drugs for salmonellosis treatment in infants [27]. However, our study revealed high rates of resistance against third-generation cephalosporins such as ceftriaxone (26.7%), which is higher than the nationwide average [5]. Hence, the rapid increase of resistance to third-generation cephalosporins in infants has become a warning signal. Not only that, the proportion of MDR isolates is up to 90.0%, far surpassing the average of Salmonella. Spp [7]. This finding is consistent with the studies by Ke B [7] which illuminate that the ratio of MDR in Salmonella Typhimurium was very high and increased yearly, even worse in infants. Hence, the increase of MDR Salmonella isolates, particularly those to ciprofloxacin and third-generation cephalosporin might lead to the failure of human infection treatments. Luckily, most of isolates (29/30) are sensitive to piperacillin/tazobactam in the study. So, we could consider piperacillin/tazobactam as an alternative medicine to treat infections caused by third-generation cephalosporin resistant strains or MDR Salmonella isolates in infants to reduce the use of carbapenem antibiotics. In our study, 16 different antimicrobial resistance patterns are observed and the most frequent resistance type was antibiotype 13 (resistance to streptomycin, tetracycline, and amoxicillin), occurring in 43.3% of the isolates. Taken together, close surveillance of Salmonella and their microbial resistance patterns is important to prevent and control the growth of MDR clones [28].

In order to evaluate the potential factors that may contribute to the ability of S. Typhimurium to cause an infection, 12 virulence genes were investigated by PCR. In the present study, all of the strains were positive for 9 virulence genes (misL, orfL, pipD, prgH, sifA, sopB, sitC, spiC, and invA), with iroN genes having a prevalence rate of 87.1%. The gene with the lower prevalence rate was sopE (22.58%). Meanwhile, the plasmid encoded fimbriae (pefA) [29] gene was found to be present in 3.33% (1/30) of the isolates. Among these virulence genes, the SPI-1 effectors (including invA, prgH, sopB, and sopE) promote invasion of human intestinal epithelial cells and initiation of enterocolitis. The SPI-2 effector (including sifA) gene is related to the promotion of intracellular growth [14]. Meanwhile, the plasmid gene (including pefA) which has been related to bacterial adhesion of intestinal epithelial cells [28]. It has been suggested that plasmids mainly play a role in systemic infection, but rarely in the gastrointestinal form and almost all of the strains with virulence plasmids are isolated from animal organs or human blood, but rarely from feces or food samples [29]. In our study, the human strains were isolated from stool samples, therefore, low presence of strains with virulence plasmids were detected. In other words, the existence of plasmid virulence genes in S. Typhimurium indicates this serotype has the possibility of causing extra-intestinal infection also. In general, a high proportion of virulence genes in these studies stress the pathogenic potential of these strains, which may play an important role in causing human salmonellosis.

Several molecular typing methods such as pulse Field Gel Electrophoresis (PFGE) [30-33], multilocus sequence typing (MLST) [30,31], and multiple-locus variable-number tandem repeat analysis (MLVA) [30,31] have been explored for molecular characterization of S. Typhimurium strains. Among these methods, PFGE is considered to be a 'gold standard' fingerprinting method for assessing relatedness among different isolates and for outbreak investigations [31]. In our study, 30 isolates exhibited genetic diversity, while no predominant PFGE patterns were observed in S. Typhimurium. There were almost no obvious relationships between antimicrobial agents and virulence patterns and PFGE clusters within these isolates. Therefore, for these organisms, a combination of different molecular typing methods could be used to identify the discrimination better [31]. But there was one exception, virulence profile VP1, which harbors pefA, showed more diversity than the other virulence profiles among PFGE profiles. However, the number of cases was too small to decide that there was a relationship between VP1 and PFGE profiles. Further observations need to be carried out.

CONCLUSION

S. Typhimurium was the most common serovar mainly causing salmonellosis in infants during the period from 2015 to 2017 in Fuzhou, China. The CDC has shown that livestock and poultry meats are an important source of S. Typhimurium in China [34], which may be transmitted to humans through the foods we eat. A key strategy to prevent foodborne infections is to improve the safety of complementary foods introduced to infants. Meanwhile, the data provided valuable information on virulence gene content and antibiotic resistance patterns in S. Typhimurium isolated from infants. Furthermore, we showed that S. Typhimurium can undergo minor genetic changes. Close surveillance of Salmonella and its microbial resistance patterns and genetic diversity is therefore important to prevent disease outbreaks, track the potential transmission routes, and determine the antimicrobial treatment of salmonellosis. However, the analysis of more strains from different regions and in combination of other molecular genotyping methods will improve our recognition of salmonella epidemiology.

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Declaration of Interest:
The authors stated that there are no conflicts of interest regarding the contents of this article.

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