Multi-locus sequence and drug resistance analysis of *Salmonella* infection in children with diarrhea in Guangdong to identify the dominant ST and cause of antibiotic-resistance

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Abstract. Multi-locus sequence typing (MLST) can be used to analyze the homology among the drug resistance gene cassettes in *Salmonella* and determine the prevalence. Information extracted using this technique can provide a theoretical basis for hospitals to devise protocols to control *Salmonella* infections. The aim of the present study was to investigate the possible association between drug resistance and integrons in clinical isolates of *Salmonella* from human fecal samples. Therefore, in the present study, 52 clinical fecal isolates of non-duplicate (i.e., not genome contamination) *Salmonella* were harvested from children with diarrhea and used for bacterial identification using biochemical tests, drug susceptibility analysis by antibiotic susceptibility testing and serotype identification using an agglutination assay. In total, seven *Salmonella* housekeeping genes (*chorismate synthase, β sliding clamp of DNA polymerase III, uroporphyrinogen-III synthase, histidinol dehydrogenase, phosphoribosylaminoimidazole carboxylase catalytic subunit, 2-oxoglutarate dehydrogenase E1 component* and *homoserine dehydrogenase*) were amplified and sequenced using MLST, before sequence alignment was performed against the Pub MLST database to determine the sequence-typed (ST) strains and construct genotypic evolutionary diagrams. Subsequently, the 52 *Salmonella* strains were subdivided into 11 serotypes and 11 sequence types. The dominant subtypes were found to be *Salmonella typhimurium* ST34 and ST19, which were diversely distributed. However, no new subtypes were found. Although the serotypes, including ST19, ST29, ST34, ST40, ST11, ST27, ST469, ST365, ST1499, ST413 and ST588, were closely associated with the MLST subtype, they did not correspond entirely. The detection rate of class I integrons was 38.46% (20/52), but no class II and III integrons were detected. The variable regions of three of 20 class I integrons were found to be amplified, whereas nine gene cassettes, including *dihydrofolate reductase A12*, *open reading frame F*, *aminoglycoside-adenylyltransferase* (*aad)A2*, *aadA22*, *aadA23*, *aadA1*, *cadmium-translocating P-type ATPase 2*, *lincosamide* and *linF,* were associated with drug resistance. These data suggest that Class I integrons are important factors underlying drug resistance in *Salmonella*, which may serve a role in the spread of drug resistance and warrant specific focus. In addition, MLST typing and serotyping should be applied cooperatively in epidemiological research.

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Introduction

Salmonella is a gram-negative, facultative, intracellular bacterium that frequently causes human and animal diseases (1). According to the WHO, >90 million people are infected by *Salmonella* annually, 150,000 of whom will succumb to *Salmonella* infection. The main clinical symptoms of *Salmonella* infection, also called salmonellosis, are sepsis and gastroenteritis (2). To date, >2,600 subtypes of *Salmonella* have been discovered and categorized (3). *Salmonella* serotypes are typically associated with their host adaptation and virulence capability, rendering serotyping to be a key tool for *Salmonella* surveillance and outbreak investigations (4). However, treatment of invasive salmonellosis has been compromised due to the emergence of *Salmonella* strains that are resistant to a

variety of first-line drugs such as ampicillin, chloramphenicol and co-trimoxazole (5). Therefore, an appropriate molecular typing method should be used in combination with serotyping to investigate the epidemiology of *Salmonella*.

Accurate typing and tracing are important for microbial epidemiological investigation, food safety and public health. Bacterial typing methods that are available include phenotyping such as phage-typing, serotyping and ribotyping and genotyping such as plasmid profile, pulsed-field gel electrophoresis and multi-locus sequence typing (6). Among them, serotyping and multi-locus sequence typing (MLST) are the most frequently used (7). Typing methods of *Salmonella* can be divided into phenotypic typing based on their phenotypic characteristics and molecular typing based on their gene expression patterns (8). Serotyping using standard agglutination methods has been the most common form of typing since 1934 (9). By contrast, molecular typing techniques mainly include pulsed field gel electrophoresis (PFGE) (10), MLST and multi-locus variable-number tandem repeat analysis (8). MLST has become a particularly popular molecular typing method due to its advantages: i) High resolution, it is easy to discover differentiation of genetically related bacterial isolates from nonambiguous sequencing data, which is superior to serotyping and/or PFGE typing (11); and ii), reproducibility and universality, MLST can be readily reproduced and does not require access to specialized reagents or training (12). In particular, *Salmonella* MLST is a molecular typing method that is based on the sequencing of seven housekeeping genes of *Salmonella,* namely *chorismate synthase* (*aroC*), *β sliding clamp of DNA polymerase III* (*dnaN*), *uroporphyrinogen-III synthase* (*hemD*), *histidinol dehydrogenase* (*hisD*), *phosphoribosylaminoimidazole carboxylase catalytic subunit* (*purE*), *2-oxoglutarate dehydrogenase E1 component* (*sucA*) *and homoserine dehydrogenase* (*thrA*) (13). These genes are highly conserved and only slowly accumulate site changes (14). *Salmonella* MLST is mainly applied to understand the hereditary backgrounds, origins and diversification of the various *Salmonella* sub-strains.

In the present study, the serotypes, MLST, drug resistance, integrin class and distribution of 52 clinical isolates of non-duplicated *Salmonella* which is not genome contamination from children with diarrhea were analyzed. Specifically, the possible correlation among the allelic profiles of the housekeeping genes *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA* and the *Salmonella* eBrust Groups (eBGs) and serotypes for potential *Salmonella* serovar prediction were focused upon. In addition, the association between drug resistance and integrin distribution of the *Salmonella* strains was investigated.

In order to aid clinicians in improving the clinical prevention and treatment of *Salmonella* infection, 1,725 diarrhea samples from children were collected to isolate the *Salmonella* strain in the present study. The drug sensitivities of those *Salmonella* strains were detected focusing on 12 different antibiotics. Total *Salmonella* genome DNA (gDNA) were extracted for MLST analysis of characteristics of *Salmonella*. After sequence analysis, eBURST analysis was applied for the various *Salmonella* sequence types (STs). The class I, II, and III integron distribution was also analyzed to map the integrated substructures of the integrons.

Materials and methods

Strain conservation. The present study was approved by the Medical Ethics Committee of Qingyuan People's Hospital, Qingyuan, China (approval no. A0051). Written informed consent was obtained from each participant's legal guardian. In total, 52 'non-duplicate' (confirmed as not a biological contamination) *Salmonella* strains were harvested from the feces of children (age, 3 months to 14 years) with diarrhea between September 2018 and April 2020 at the Department of Clinical Laboratory, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Qingyuan, China. Patients aged ≤14 years who were admitted to the Outpatient Department or hospitalized at Qingyuan People's Hospital due to diarrhea were selected as candidates. A total of 1,725 children (890 male and 835 female children, mean age 5.6±0.41, median age 5.1±0.69) were enrolled in our study, all of whom exhibited one or more of the following clinical manifestations: Diarrhea, fever and/or abdominal pain. The exclusion criteria were: i) Children with diarrhea presenting with watery stools, without red blood cells, white blood cells or pus cells in routine stool test; ii) with low complete blood count; and iii) with mild symptoms. The quality control strains used for antibiotic susceptibility testing were *Pseudomonas aeruginosa* (ATCC.27853), *Staphylococcus aureus* (ATCC.25923) and *E. coli* (ATCC.25922) (all from American Type Culture Collection) (15). The positive integron reference strain was a clinical isolation *Klebsiella pneumoniae* strain (1162281; GSK plc.) which contained Class I, II, and III integrons (16).

Instruments and reagents

Instruments. BD Phoenix™ M50 automatic detector and bacterial drug sensitivity analysis system (BD Biosciences). NMIC/ID-4 composite board for the drug sensitivity tests (BD Biosciences). Thermal Cycler T100 (Bio-Rad Laboratories, Inc.). Gel Dox™ XR+ gel imaging system (Bio-Rad Laboratories, Inc.).

Reagents. Triple sugar iron (TSI) agar medium (Qingdao Hope Bio-Tcehnology Co.,Ltd). Bacterial group DNA extraction kit (ab288102, Abcam), 2X Taq PCR MasterMix (KT121221, Tiangen Biotech Co., Ltd.), Gel Red nucleic acid dye (SCT123, Sigma-Aldrich; Merck KGaA), and Marker I, II and III DNA ladder (MD101, MD102 and MD103; Tiangen Biotech Co., Ltd.). Primers (Sangon Biotech Co., Ltd.).

Methods

Sample collection and bacterial culture. Fecal samples from children with diarrhea were collected for *Salmonella* isolation and identification. A small amount (10-50 mg) of feces from each child with diarrhea was collected using sterile swabs, which were placed in 9 ml selenite brilliant green sulfa enrichment broth (E-MA73; Eiken Chemical Co., Ltd.) and cultured at 36˚C for 18 h. After enrichment, 10 μl of the samples was streaked onto a *Salmonella* chromogenic medium agar plate (CM1007B; Thermo Fisher Scientific, Inc.), before further culture at 36˚C for 18 h. Suspected colonies that are purplish red or wine red with a diameter of 2-3 mm were selected from the plate for biochemical identification.

Table I. Primer sequences used for multi-locus sequence typing.

Gene	Oligonucleotide and sequence (5'-3')	Length/bp
Chorismate synthase	F: CCTGGCACCTCGCGCTATAC R: CCACACACGGATCGTGGCG	826
β sliding clamp of DNA polymerase III	F: ATGAAATTTACCGTTGAACGTGA R: AATTTCTCATTCGAGAGGATTGC	833
Uroporphyrinogen-III synthase	F: GAAGCGTTAGTGAGCCGTCTGCG R: ATCAGCGACCTTAATATCTTGCCA	666
Histidinol dehydrogenase	F: GAAACGTTCCATTCCGCGCAGAC R: CTGAACGGTCATCCGTTTCTG	894
Phosphoribosylaminoimidazole carboxylase catalytic subunit	F: ATGTCTTCCCGCAATAATCC R: TCATAGCGTCCCCCGCGGATC	510
2-oxoglutarate dehydrogenase E1 component	F: AGCACCGAAGAGAAACGCTG R: GGTTGTTGATAACGATACGTAC	643
Homoserine dehydrogenase	F: GTCACGGTGATCGATCCGGT R: CACGATATTGATATTAGCCCG	852

F, forward; R, reverse.

Serotyping by slide agglutination. *Salmonella* enterica isolates were cultured at 37˚C overnight. A drop of broth was dropped on a glass slides to test somatic O antigen by slide agglutination. Meanwhile, each *Salmonella* strain was grown on Swarm agar plates at 37˚C overnight, and single colonies were picked to test phases 1 and 2 of H antigens by slide agglutination. Diagnostic sera for *Salmonella* antigens were purchased from Tianrun Bio-Pharmaceutical Co. Ltd. and S&A Reagents Lab Ltd. Serotyping and biotyping were performed according to the modified Kauffmann-White scheme, which is a modification of the original scheme from the 1930s (7).

Identification of Salmonella and drug sensitivity detection. All *Salmonella* strains were inoculated onto TSI agar plates and cultured for 12-18 h at 37˚C. Antibiotic susceptibility test (AST) was performing using the BD Phoenix™ M100 Automated Microbiology System (BD Biosciences), a broth-based microdilution method that utilizes a redox indicator (colorimetric-oxidation-reduction) to enhance the detection of bacterial growth. AST was performed on BD Phoenix™ NMIC-502 panels (BD Biosciences). Samples were prepared and experimental conditions were set following the manufacturer's protocols. Phoenix ID broth (4 ml) was first inoculated with bacterial colonies from a pure culture adjusted to a 0.5 McFarland standard using a CrystalSpec nephelometer (BD Biosciences), before the suspension was poured into the ID side of the Phoenix panel. Bacterial growth at 37˚C in the panels was then monitored every 20 min to determine minimal inhibitory concentration (MIC) of antibiotics. The interpretation of MIC results were recorded as either susceptible (S), intermediate (I) and resistant (R) according to the 2016 CLSl M100-S26 susceptibility test guide (17). Drug resistance rate=(susceptible strain number/total *Salmonella* strain number) x100%.

In total, 12 different antibiotics were used base on the following antibiotic classification: i) Class A, including carbenpenems imipenem and meropenem; ii) class C, such as quinolones moxifloxacin; iii) class D, including cephalosporins cefotaxime and cefepime; iv) class E, such as broad-spectrum penicillins ampicillin, ampicillin/sulbactam, piperacillin and piperacillin/tazobactam; v) class F, including β-lactam/β-lactamase inhibitor combinations, including amoxicillin, amoxicillin/clavulanate and aztreonam; and vi) class G, including phenicols chloramphenicol.

Total bacterial DNA extraction. *Salmonella* genomic DNA was extracted in accordance with the protocol of the Bacterial group DNA extraction kit (ab288102; Abcam), transferred to an aseptic 1.5 ml tube and stored at -80˚C.

Primer design. In total, seven housekeeping genes of *Salmonella*, *aroC, dnaN, hemD, hisD, purE, sucA* and *thrA*, were chosen for MLST analysis (18). The primer sequences were designed according to the *Salmonella* MLST database (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-senterica.html) and are listed in Table I. Primers for the *intI1*, *intI2*, *intI3* gene and variable region were designed using primer3Web (version 4.1.0; http://primer3.ut.ee/) as described previously (19) and are listed in Table II.

PCR for Salmonella MLST, integrase genes and variable regions. The PCR mix for the seven housekeeping genes was constructed as follows: 25 μl 2X Taq PCR MasterMix, 1 μl each of the forward and reverse primers, 2 μl *Salmonella* gDNA template and 22 μl ddH_2O. The reaction conditions were as follows: Initial denaturation at 94˚C for 5 min; followed by 32 cycles of denaturation at 95˚C for 30 secs, annealing at 60˚C for 1 min and extension at 72˚C for 1 min; and a final extension at 72˚C for 10 min. The PCR products were visualized electrophoretically on an agarose gel using Gel Red nucleic acid dye (Sigma-Aldrich; Merck KGaA) and sent to Sangon Biotech Co., Ltd. for bidirectional Sanger sequencing.

The PCR mix for the class I, II, and III integrons comprised the following: 12.5 μl 2X Taq PCR MasterMix, 0.5 μl each

Table II. Primer sequences of the three integrase genes and variable areas.

Target gene	Oligonucleotide (5'-3')	Length/bp
Int 1	F: GGTCAAGGATCTGGATTTCG R: ACATGCGTGTAAATCATCGTC	493 bp
Int 2	F: CACGGATATGCGACAAAAAGGT R: GTAGCAAACGAGTGACGAAATG	789 bp
Int 3	F: AGTGGGTGGCGAATGAGTG R: TGTTCTTGTATCGGCAGGTG	922 bp
Int-variable area	5'-CS: GGCATCCAAGCAGCAAG 3'-CS: AAGCAGACTTGACCTGA	variable

F, forward; R, reverse; CS, conserved segment; *int*, integrase gene.

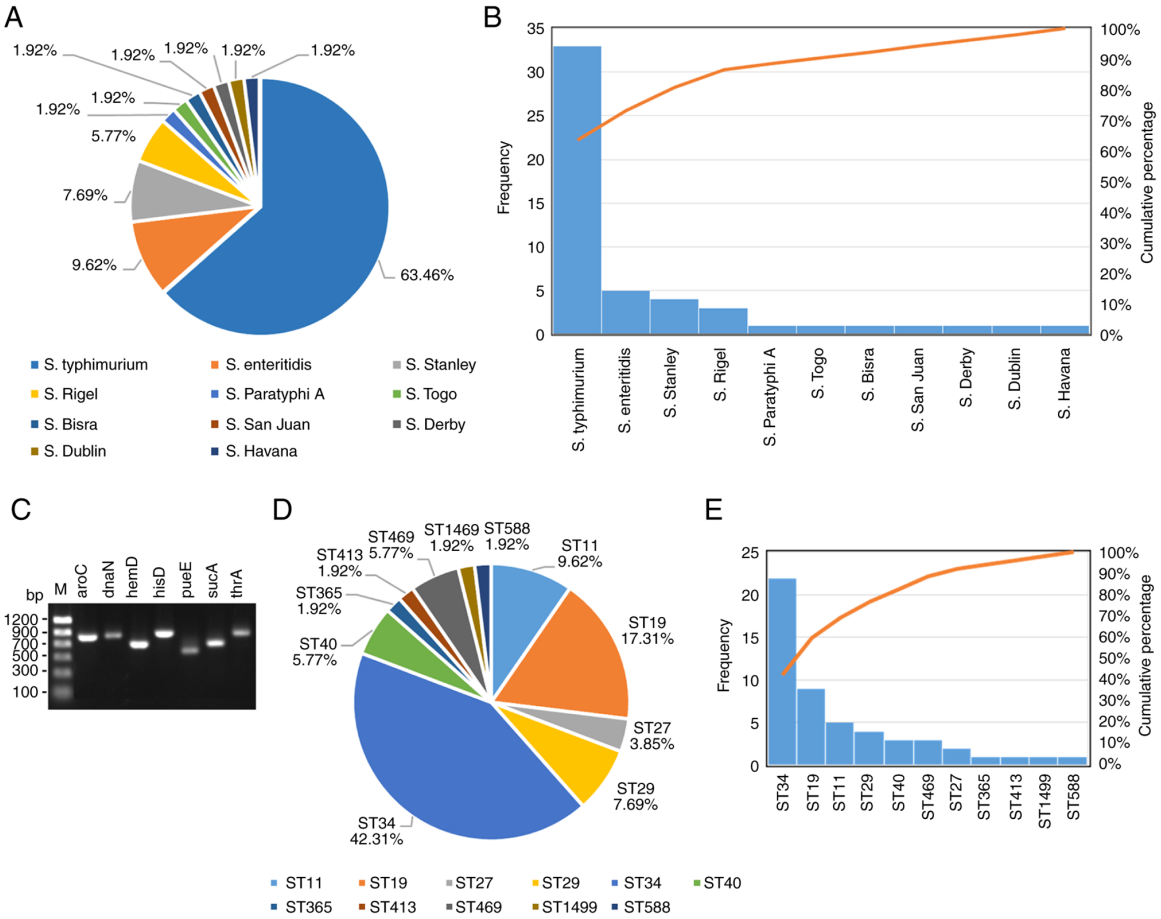

Figure 1. Serotype and ST distribution of *Salmonella* strains in the present study. (A) Serotype distribution of the 52 *Salmonella* strains in the present study. (B) Pareto chart of the serotypes of 52 *Salmonella* strains in the present study. (C) Seven pairs of housekeeping genes as determined using PCR-based multi-locus sequence typing. (D) ST distribution of 52 *Salmonella* strains in the present study. (E) Pareto chart of ST of 52 *Salmonella* strains in the present study. ST, sequence type; M, marker; aroC, chorismate synthase; dnaN, β sliding clamp of DNA polymerase III (dnaN); hemD, uroporphyrinogen-III synthase; hisD, histidinol dehydrogenase; purE, phosphoribosylaminoimidazole carboxylase catalytic subunit; sucA, 2-oxoglutarate dehydrogenase E1 component; thrA, homoserine dehydrogenase.

of the forward and reverse primer, 0.5 μl *Salmonella* gDNA template and 11.5 μl ddH_2O. The reaction conditions were as follows: Initial denaturation at 95˚C for 5 min; followed by 26 cycles of denaturation at 94˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 1 min; and a final extension at 72˚C for 5 min. In total, 5 μl PCR products were loaded

Table III. *Salmonella* multi-locus sequence typing and distribution rate in 52 samples.

Chorismate synthase	β sliding clamp of DNA polymerase III	Uroporphyrinogen-III synthase	Histidinol dehydrogenase	Phosphoribosylaminoimidazole carboxylase catalytic subunit	2-oxoglutarate dehydrogenase E1 component	Homoserine dehydrogenase	Sequence types	Distribution rate %
5	2	3	7	6	6	11	11	9.61
2	59	23	64	38	19	12	1499	1.92
10	7	12	9	5	9	2	19	17.31
5	14	79	9	6	12	17	27	3.85
186	35	78	75	39	182	97	588	1.92
16	16	20	18	5	12	18	29	7.69
10	19	12	9	5	9	2	34	42.31
130	97	25	125	84	9	101	365	1.92
19	20	3	20	5	22	22	40	5.77
15	70	93	78	113	6	68	413	1.92
92	107	79	156	64	151	87	469	5.77

onto a 2% agarose gel (60 min, 80 V) and visualized using Gel Red nucleic acid dye and a gel imaging system (Bio-Rad Laboratories, Inc.).

The PCR mix for the variable regions of class I, II, and III integrons comprised the following: 25 μl 2X Taq PCR MasterMix, 0.5 μl each of the forward and reverse primer, 1 μl DNA template and ddH_2O to 50 μl. The reaction conditions were as follows: Initial denaturation at 94˚C for 5 min; followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 2 min; and a final extension at 72˚C for 7 min. PCR products (5 μl) were visualized electrophoretically on a 1.2% agarose gel (60 min, 80 V) using Gel Red nucleic acid dye and a gel imaging system (Bio-Rad Laboratories, Inc.).

Sequence analysis of the variable regions. The PCR products (displaying bright bands) were sent to Sangon Biotech Co., Ltd. for sequencing. Corrected sequencing data were obtained by removing failed signals with Chromas software (version 2.6.6; http://technelysium.com.au/wp/chromas/). Subsequently, sequencing data were compared and analyzed using BLAST (BLAST+ version 2.10.0; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Results with 100% coincidence were selected.

Genotyping analysis of Salmonella MLST. The sequencing reads were subjected to the *Salmonella* MLST database v1.1.3 (http://mlst.warwick.ac.uk/mlst/dbs/Senterica), before the different allele values were obtained by allele/ST query to identify the corresponding ST, yielding an allelic spectrum. The ST classification data were then uploaded onto the Pub MLST Data Analysis (http://pubmlst.org/salmonella/) tool to conduct eBURST analysis to obtain the BURST group diagram (group definition: Profiles match at n-2 loci to any other member, 'n' is the number of loci in the scheme). The standard of clustering used is that if four subunits in seven ST-labeled genes are the same, then they would be considered to be in the same composite clone group (20,21).

Statistical analysis. All statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc.). Drug resistance was analyzed using the $\chi 2$ test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serotyping. A total of 11 serotypes were obtained from the 52 *Salmonella* strains, including 33 strains of *S. Typhimurium*, five strains of *S. Enteritidis*, four strains of *S. Stanley*, three strains of S. *Rigel* and one strain each of *S. Paratyphi A, S. Derby, S. Dublin, S. San Juan, S. Togo, S. Bisra* and *S. Havana* (Fig. 1A and B).

MLST results. To estimate the genetic correlations, MLST was performed. In total, seven housekeeping gene loci, namely *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*, were chosen for MLST analysis of *Salmonella* (Fig. 1C). A total of 52 *Salmonella* strains were divided into 11 STs base on MLST result (Table III), including 22 strains with ST34 (42.31%), nine strains with ST19 (17.31%), five strains with ST11 (9.62%), four strains with ST29 (7.69%), three strains with ST40 (5.77%) and ST469 (5.77%), two strains with ST27 (3.85%) and one strain with ST365 (1.92%), ST413 (1.92%), ST1499 (1.92%) and ST588 (1.92%) (Fig. 1D and E).

Table IV. *Salmonella* ST type corresponding to each serotype in 52 samples.

Serotypes	ST types (number of strains)
Salmonella Typhimurium	ST19 (9), ST29 (2), ST34 (21), ST40 (1)
Salmonella Enteritis	ST11 (4), ST40 (1)
Salmonella Stanley	ST29 (2), ST27 (2)
Salmonella Rigel	ST469 (3)
Salmonella Paratyphi A	ST365 (1)
Salmonella Togo	ST34 (1)
Salmonella Bisra	ST1499 (1)
Salmonella San Juan	ST413 (1)
Salmonella Derby	ST40 (1)
Salmonella Dublin	ST11 (1)
Salmonella Havana	ST588 (1)

ST, sequence type.

Association between MLST and serotyping. Among the 52 *Salmonella* samples, each *Salmonella* serotype corresponded to ≥ one MLST type. The most common serotype and ST type were *S. Typhimurium* (33/52) and ST34 (22/52), respectively. These two types are the most prevalent in the South of China. The three other predominant types were *S. Enteritideis*, *S. Stanley* and *S. Rigel*, accounting for ~80.77% in total. In the aforementioned serotypes, four strains of ST11, nine strains of ST19, two strains of ST27, four strains of ST29, 21 strains of ST34 and two strains of ST40 were identified, where ST34 was the most abundant ST within the *S. Typhimurium* subtype (63.63%), whereas ST11 was the most abundant ST of *S. Enteritidis* (80%; Table IV).

Analysis of the similarity, variability and evolutionary relationships among different ST types of Salmonella. eBURST is an algorithm that can identifies groups of closely associated sequence types from MLST data (20). It was used to analyze the possible similarity, variability and evolutionary relationships among different ST types of *Salmonella* in the present study. The genetic backgrounds were found to be diverse among the STs identified in the present study. In total, 11 STs belonged to 10 different eBURST groups (eBGs).

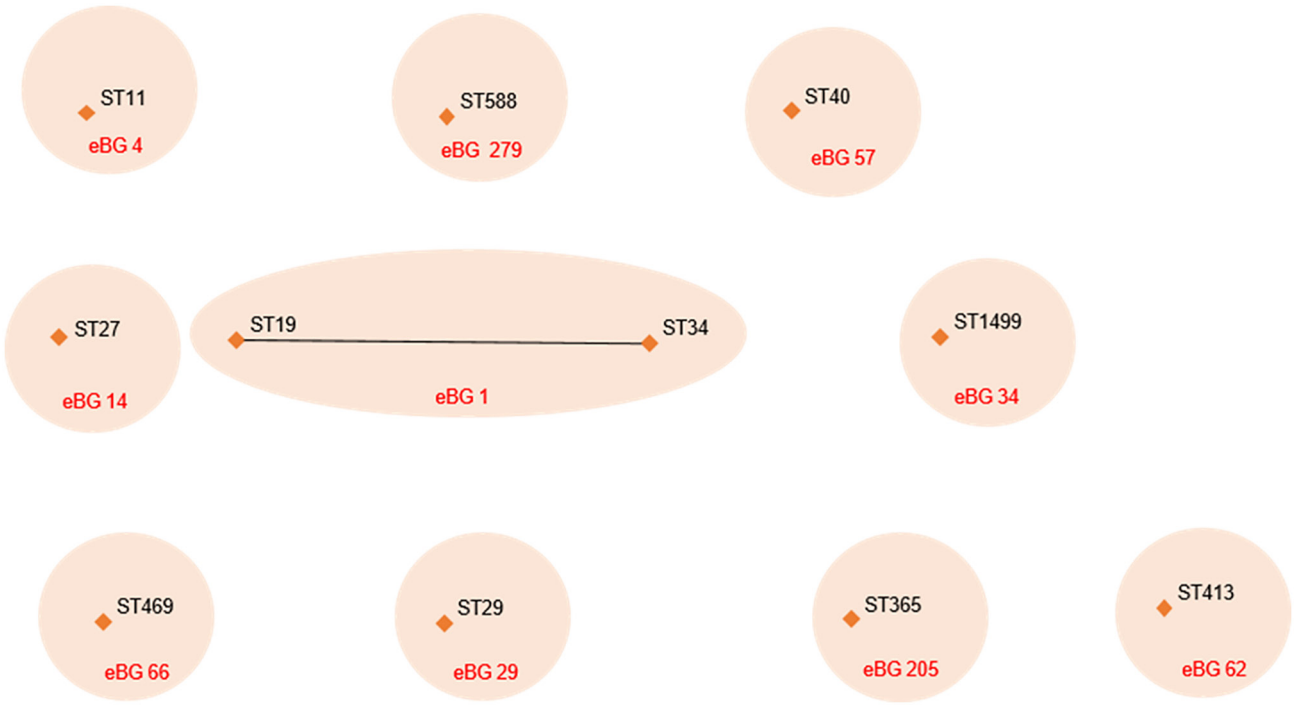

Figure 2. eBURST diagram for the STs. eBURST was used to analyze the similarity, variability and evolutionary relationships among the different ST types of *Salmonella*. The eBURST diagram was generated using the STs of 52 *Salmonella* samples in the present study. ST19 and ST34 formed a composite group (eBG1) with a high genetic relationship. ST, Sequence type; eBG, eBURST group.

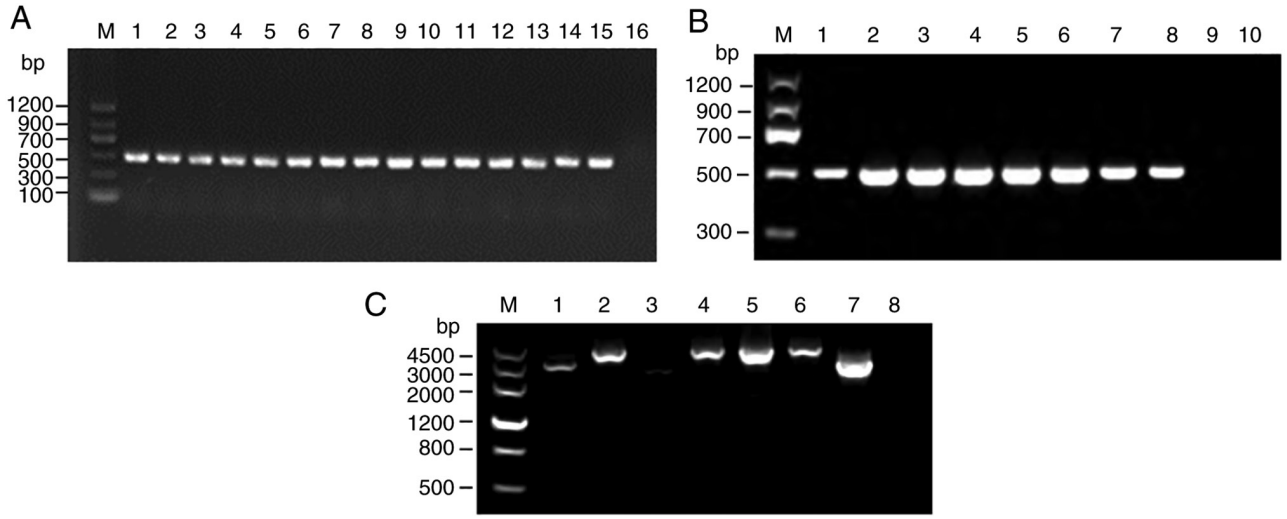

Figure 3. PCR for integrin and multi-locus sequence typing. (A and B) In total, 20 strains harboring the class I integrase gene following PCR. (A) Lane 2-15 and (B) lane 3-8 represent class I integron-positive samples. Lane 1 in (A) and lanes 1 and 2 in (B) represent positive controls (*Klebsiella pneumonia* gDNA as template). Lane 16 in (A) and lane 9 in (B) are negative controls (*E. coli* ATCC 25922 gDNA as template). Lane 10 in (B) is a blank control. (C) Variable regions in nine strains revealed through PCR analysis. Lanes 1 and 2 represent positive controls (*Klebsiella pneumonia* gDNA as template), Lanes 4-7 represent variable region-positive samples. Lane 3 represent negative control (*E. coli* ATCC 25922 gDNA as template). Lane 8 represents blank control.

Further analysis also indicated that ST19 and ST34 are part of the same composite group (eBG1) sharing a high genetic relationship (Fig. 2).

Association between class I integrons and anti-bacterial resistance and anti-microbial resistance profile. Among the 52 *Salmonella* strains, 20 harbored class I integrase, where the detection rate was 38.46% (20/52). However, none of the 52 strains harbored class II or III integrons (Fig. 3A and B). The resistance rates of strains harboring class I integrons toward ampicillin, ampicillin/sulbactam, chloramphenicol and moxifloxacin were found to be significantly higher compared with those of class I intergroup-negative strains ($P<0.05$). Neither class I integron-positive nor class I integron-negative strains were resistant to amoxicillin/clavulanate, meropenem, piperacillin/tazobactam or imipenem. The resistance rates of class I integron-positive strains to aztreonam, piperacillin, cefepime and cefotaxime were increased compared with those of class

Table V. Drug resistance phenotypes between 52 *Salmonella* profile I integron-positive and -negative strains.

		Profile I integron positive strains (n=20)			Profile I integron negative strains (n=32)			
Antibiotic	DRR (%)	DRR (%)	IR (%)	SR (%)	DRR (%)	IR (%)	SR (%)	P-value[a]
Amoxicillin/Clavulanate	0.0	0.0	13.3	86.7	0.0	0.0	100.0	-
Ampicillin	51.9	75.0	0.0	25.0	37.5	0.0	62.5	<0.05
Ampicillin/Sulbactam	19.2	40.0	35.0	25.0	6.25	28.1	65.6	<0.05
Aztreonam	13.5	15.0	0.0	85.0	12.5	0.0	88.5	>0.05
Chloramphenicol	34.6	70.0	0.0	30.0	12.5	3.1	84.4	<0.05
Meropenem	0.0	0.0	0.0	100.0	0.0	0.0	100.0	-
Moxifloxacin	3.8	10.0	30.0	60.0	0.0	9.4	90.6	<0.05
Piperacillin	48.1	65.0	10.0	25.0	37.5	3.1	59.4	<0.05
Piperacillin/Tazobactam	0.0	0.0	0.0	100.0	0.0	0.0	100.0	-
Cefepime	14.0	20.0	0.0	80.0	10.7	0.0	89.3	<0.05
Cefotaxime	17.3	25.0	0.0	75.0	12.5	0.0	87.5	<0.05
Imipenem	0.0	0.0	5.0	95.0	0.0	12.5	87.5	-

[a]$\chi 2$ test. DRR, Drug resistance rate; IR, Intermediary rate; SR, sensitivity rate.

Table VI. Comparison between drug resistance phenotypes and variable region gene boxes.

Sample number	Resistance phenotype[a]	Size of the variable area (bp)	Combination of the variable region gene box
19	b, d, e, f	2800	*dfrA12, orfF and aadA2*
35	b, d, e, h	2600	*dfrA17 and aadA5*
41	b, d, e, g, h	3000	*aadA2, aadA22, aadA23 aadA1, cadA2 and lnuF, linF*

[a]Divided into nine categories: Class a is carbocylase dilutes, including imipenem and meropenem (MEM); class b is aminoglycosides, such as gentamicin and amikacin; class c is quinolones, such as ciprofloxacin, levofloxacin and ciprofloxacin; class d is cephalosporins, including cefazoxime, ceftazidime, cefotaxime and cefepime; class e is a broad-spectrum penicillin, such as ampicillin/sulbactam, piperacillin/tazobactam, ampicillin and piperacillin; class f is β-lactam, including aztreonam and amoxicillin/clavicularulanic acid; class g is sulfonamides, such as compound neomin (SXT); class h represents tetracyclines; and class i represents amides and alcohols, including chloramphenicol. Dfr, dihydrofolate reductase; Orf, open reading frame; aad, aminoglycoside-adenylyltransferase; cadA2, cadmium-translocating P-type ATPase 2; InuF, lincosamide.

I integron-negative strains, but no significance were found (Table V).

Drug resistance gene cassette distribution of class I integron-positive strains. A total of three class I integrin-positive strains and one class I integron-negative strain (four strains in total) were sent for genome sequencing (Fig. 3C), where 12 drug resistance gene cassettes were detected (Table VI; Fig. 4). The actual drug resistance genes of the successfully sequenced strains and the variable regions in the gene cassette are summarized in Fig. 4. The integrated substructure is presented in Fig. 4. The drug resistance rates of *Salmonella* class I integrin-positive strains against ampicillin, ampicillin/sulbactam, chloramphenicol, moxifloxacin, piperacillin, cefepime and cefotaxime were significantly higher than those of the negative strains (P<0.05), indicating that the mechanism underlying the acquisition of drug resistance in *Salmonella* was closely related with the presence of class I integrons (Table V). However, the resistance rates of class I integron-negative *Salmonella* strains to aztreonam, moxifloxacin were similar to class I integrin-positive strains (Table V), indicating that multidrug resistance among *Salmonella* strains is not only associated with class I integrons but also with other drug resistance mechanisms which needs further investigation.

Discussion

Salmonella is a highly versatile pathogen that can infect a wide range of hosts and cause different clinical manifestations (22). To date, >2,600 *Salmonella* serotypes have been reported worldwide, of which 292 different serotypes belonging to 35 different somatic (O) groups have been reported in China (23). *Salmonella* infections in children with diarrhea in Guangdong have been reported to be mainly caused by *S. Typhimurium*, *S. Enteritis* and *S. Stanley*, which belong to five separate O groups (24). However, human infections caused by non-typhoid *Salmonella* are becoming a global public sanitation problem, leading to ~93.8 million cases of gastroenteritis and 155,000 deaths every year (25).

In recent years, non-typhoid *Salmonella* is emerging as one of the main pathogens in infants in China, causing diarrhea, fever and abdominal pain (26-29). Children have immature immune systems and weak gastrointestinal systems that are

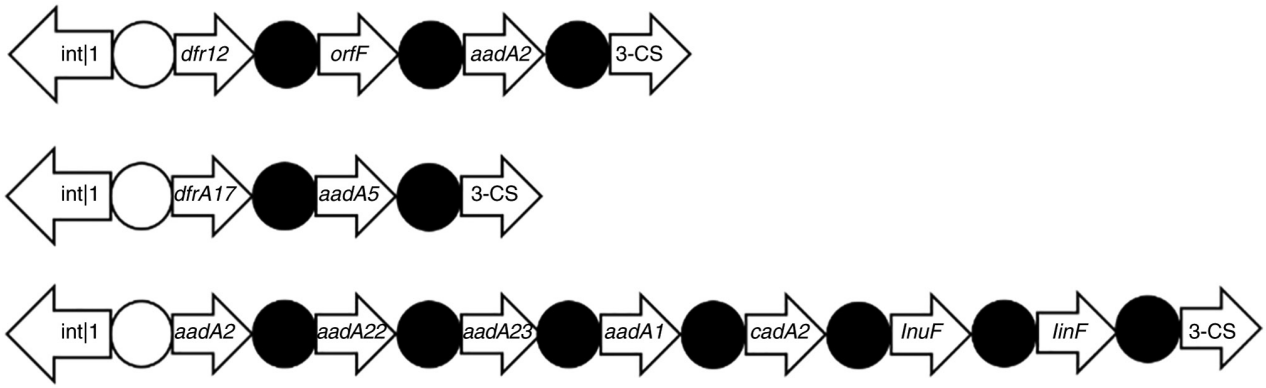

Figure 4. Integrated substructures of the integrons in the present study. Among the 52 *Salmonella* strains, 20 harbored class I integrases, where the detection rate was 38.46% (20/52), but none of the 52 strains harbored class II or class III integrons. White circle, integration site attI; black circle, integration site attC. CS, conserved sequences; Dfr, dihydrofolate reductase; Orf, open reading frame; aad, aminoglycoside-adenylyltransferase; int, integrase; cadA2, cadmium-translocating P-type ATPase 2; InuF, lincosamide.

particularly susceptible to *Salmonella* (30). In the present study, all *Salmonella* strains were collected from the fecal specimens of children with diarrhea. *S. Typhimurium* was found to be the most common serotype in the present study, with a detection rate of 63.46% (33/52), followed by *S. Enteritis* and *S. Stanley*, with detection rates of 9.62 and 7.69%, respectively.

Quinolones and third-generation cephalosporins are currently the first-line therapeutic options for the treatment of non-typhoid *Salmonella* infections (31). Due to the limitations of drug administration to children, quinolones and aminoglycosides are restricted in use (32). Therefore, third-generation cephalosporin is the priority method for the treatment of children with *Salmonella* infections (33). In the present study, drug sensitivity results showed that the resistance rate of non-typhoid *Salmonella* strains was as high as 51.9% to ampicillin, >48.1% to piperacillin and certainly >17.3% to cefotaxime. These resistance rates are similar to those reported by a previous study (34). The isolated *Salmonella* strains had drug resistance to 3rd- or 4th-generation cephalosporins. However, amoxicillin/clavulanic acid-, piperacillin/tazobactam-, imipenem- and meropenem-resistant strains could not be detected in the present study. The treatment of infectious diarrhea should be combined with drug sensitivity testing to avoid the risk of multiple drug resistance strains arising due to the overuse of clinical antibiotics.

Integrons are important genetic factors in the capture, integration and expression of drug resistance genes and are particularly abundant in Gram-negative bacteria (35,36). Integrons can carry ≥ one drug resistance gene cassettes, which is an important mechanism of the horizontal transmission of drug resistance genes (37-39). In the present study, class I, II and III integrons and variable regions were sequenced and analyzed in the 52 non-typhoid *Salmonella* strains. In total, 20 strains harbored class I integrons with a positive rate of 38.46%, compared with the 57.0% reported by a previous study (40). The prevalence of integrons found in *Salmonella* varies from country to country and depends on the origin of the isolates (41). There have been several reports associating the prevalence of Class I integrons in *Salmonella* isolates from different places in China. Lu *et al* (42) reported that 66.5% class I integrons in *Salmonella enterica serovar Indiana* (87.2%) and *Enteritidis* (50.8%) were isolated from chicken samples in Eastern China. In addition, class I integrons were detected in 26.9% of the broiler chicken in Shandong, China (43), whilst 34.7% class I integron-positive *Salmonella* were isolated in duck farms and in a slaughterhouse in Shandong province, China (44). However, another previous study reported only 16.9% positivity in terms of class I integrons in *Salmonella* isolated from farm animals in Shandong province, China (45). Zhang *et al* (46) also reported that 17.4% *Salmonella* isolated from healthy humans were positive for class I integrons in Guangdong in China.

In the present study, 12 types of drug resistance gene cassettes were detected, namely *dfrA12*, *orfF*, *aadA2*, *drfA17*, *aadA5*, *aadA2*, *aadA22*, *aadA23*, *aadA1*, *cadA2*, *InuF* and *linF*. However, Class II and III integrons could not be detected in the present study. The class I integron-positive strain antibiotic resistance rate was found to be significantly higher compared with that of the integron-negative strains, except for sensitivity to amoxicillin/clavulanic acid, piperacillin/tazobactam and imipenem, according to the drug susceptibility analysis. These results suggest that integron gene-positive strains are associated with significant multidrug resistance, consistent with previous reports (47,48). Class I integrons greatly increase the risk of horizontal drug resistance gene transmission due to their mobile and integration features (49). The cautious use of antimicrobial agents is essential for preventing the emergence and spread of drug-resistant strains of bacteria (50). The integron-positive and antibiotic-resistant genes found in the *Salmonella* isolates in the present study may contribute to the control and therapy of *Salmonella infection*. However, further studies are necessary to determine the significance of class I integron in the distribution of antibiotic resistance.

MLST is a high-resolution typing technique first proposed by Maiden *et al* (12) in 1998, which was developed based on the technique of multi-site enzyme electrophoresis. Harbottle *et al* (51) then used PFGE and MLST to type 81 strains of *Salmonella enteritis*, indicating that MLST was a suitable technique for the typing of the different *Salmonella* serotypes. Although MLST has advantages that can potentially replace and supplement serotyping, the principle of MLST is different from that of serotype detection. The combination of

MLST and serotype detection can facilitate research on the hereditary and evolutionary relationships of *Salmonella*. The most common *Salmonella* sequence types were found to be ST19 and ST34 in the present study, where the corresponding serotype was *S. Typhimurium*. This is consistent with findings from a previous report, where the most prevalent *Salmonella* subtypes found were ST34 and ST19 in the Guangdong province in 2007-2011 (52). Observations from the present study therefore provided important evidence and confirmed further that these two types of *Salmonella* can serve an important role in pediatric diarrhea in Guangdong, China. Since the drug resistance characteristics of predominant *Salmonella* ST and genetic drift between various *Salmonella* ST could guide the clinical antibiotic treatment of *Salmonella* infection and epidemics, the present study may lay a foundation for a therapeutic strategy for pediatric diarrhea caused by *Salmonella* infection in the future.

The characteristics of *Salmonella* infection differ depending on the region. *S. paratyphoid A* tended to dominate in Yunnan from 1995 to 2013, where the dominant sequence types were ST85 and ST129 (53). By contrast, the prevalent sequence types were found to be ST11 and ST34 in Nanjing in 2014-2015 (54). In the present study, ST34 corresponded to *S. Typhimurium* and *S. Togo*, while ST29 corresponded to *S. Stanley* and *S. Typhimurium*. In addition, although ST34 and ST29 were two different sequence types, they both belong to the serotype group B (13), suggesting that these strains may have been subjected to convergent evolution and are variations of the same strain. According to eBURST cluster analysis, ST19 and ST34 were highly associated, with only one pair of housekeeping genes that were different. Among the housekeeping genes, *thrA* comprised the largest number of alleles in the seven groups of the ST types of 52 *Salmonella* strains. By contrast, the least common was *sucA*, suggesting that *sucA* was the most stable.

The present study suggests that multi-drug resistance is closely associated with the presence of class I integrons in isolated *Salmonella* strain. the isolated *Salmonella* strains are particular resistant to ampicillin (51.9%), chloramphenicol (34.6%) and piperacillin (48.1%). This is consistent with results from a previous study conducted in Guangdong, China (52). This finding may facilitate the design of *Salmonella* antibiotics for clinical practice. In particular, since class I integrons appear to serve an important role in the acquisition of drug resistance, they warrant immediate attention. Furthermore, the present data show that MLST can be used for clinical *Salmonella* genotyping. Due to the rapid emergence of multi-drug resistance in *Salmonella*, further studies are required to investigate the mechanism underlying bacterial drug resistance, strictly monitor susceptible factors, control bacterial drug resistance, strengthen disinfection and isolation methods in clinical practice.

However, the present study remains associated with a number of limitations. The number of cases examined in the present study is small. In addition, the characterization of multi-drug resistance was not performed, where the mechanism of antibiotic resistance, virulence and transfer of resistance genes were evaluated in the present study. The sensitivity and specificity of the results of genotyping analysis of *Salmonella* MLST were also not considered.

To conclude, *Salmonella* infection in children with diarrhea was mainly caused by *S. Typhimurium*, where the most prominent sequence types were ST34 and ST19. The class I integrons found were closely associated with *Salmonella* drug resistance. Deepening the research into integrons is expected to serve an important role in understanding the occurrence and transmission mechanism of *Salmonella* drug resistance. Particular attention should be given to the 3rd- and 4th-generation cephalosporin resistance of *Salmonella*.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LX and QH performed the molecular genetic studies and participated in the sequence alignment. YT and WW performed species identification. LC and YL performed the antibiotics susceptibility tests. YT and WW performed the PCR. CY and BF conceived the study and participated in its design and coordination. LX and BF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Qingyuan People's Hospital (Qingyuan, China). Written informed consent was obtained from each participant's legal guardian.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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