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New Clustered Regularly Interspaced Short Palindromic Repeat Locus Spacer Pair Typing Method Based on the Newly Incorporated Spacer for *Salmonella enterica*

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A clustered regularly interspaced short palindromic repeat (CRISPR) typing method has recently been developed and used for typing and subtyping of *Salmonella* spp., but it is complicated and labor intensive because it has to analyze all spacers in two CRISPR loci. Here, we developed a more convenient and efficient method, namely, CRISPR locus spacer pair typing (CLSPT), which only needs to analyze the two newly incorporated spacers adjoining the leader array in the two CRISPR loci. We analyzed a CRISPR array of 82 strains belonging to 21 *Salmonella* serovars isolated from humans in different areas of China by using this new method. We also retrieved the newly incorporated spacers in each CRISPR locus of 537 *Salmonella* isolates which have definite serotypes in the Pasteur Institute's CRISPR Database to evaluate this method. Our findings showed that this new CLSPT method presents a high level of consistency (kappa = 0.9872, Matthew's correlation coefficient = 0.9712) with the results of traditional serotyping, and thus, it can also be used to predict serotypes of *Salmonella* spp. Moreover, this new method has a considerable discriminatory power (discriminatory index [DI] = 0.8145), comparable to those of multilocus sequence typing (DI = 0.8088) and conventional CRISPR typing (DI = 0.8684). Because CLSPT only costs about $5 to $10 per isolate, it is a much cheaper and more attractive method for subtyping of *Salmonella* isolates. In conclusion, this new method will provide considerable advantages over other molecular subtyping methods, and it may become a valuable epidemiologic tool for the surveillance of *Salmonella* infections.

Microbiologists have used serological and nutritional characteristics to subdivide pathogenic bacteria for nearly 100 years (1). Traditional serotyping according to the White-Kauffmann-Le Minor scheme, based on the agglutination of bacteria with specific sera, identifies somatic (O) and flagellar (H) antigens (2). However, although traditional serotyping has been widely used, it still has some drawbacks. First, traditional serotyping takes at least 3 days to complete (3) and requires the maintenance of more than 250 typing sera and 350 different antigens. Second, traditional serotyping does not provide a discriminatory level sufficient for the investigation of outbreaks of food-borne illness and cannot be used to infer phylogenetic relationships.

Differentiation between isolates within the most common serotypes requires the use of subtyping methods. DNA-based subtyping methods have been developed. These include multilocus sequence typing (MLST), which requires the sequencing of seven housekeeping genes, and pulsed-field gel electrophoresis (PFGE). The latter technique is based on analysis of the restriction pattern of high-molecular-weight DNA digested with a rare restriction enzyme (4). However, both methods have several limitations. MLST is highly expensive and has low throughput (5), and PFGE is a technically demanding and nonautomated method. Furthermore, the interpretation and comparison of banding profiles is not straightforward, even with standard protocols and BioNumerics specialized analysis software (Applied Maths).

The clustered regularly interspaced short palindromic repeat (CRISPR) that has been discovered in archaea and bacteria provides adaptive, heritable immunity against viruses, plasmids, and other mobile genetic elements (6). CRISPRs encode tandem sequences containing 21- to 47-bp direct repeats (DRs) and spacers of similar size. The spacers are short DNA sequences obtained from foreign nucleic acids, such as phage or plasmids, inserted into bacterial chromosomes to protect them from infection by homologous phage or plasmids (7). As such, different CRISPRs arise due to diverse phage and plasmid pools in an environment. Thus, CRISPRs differentiate outbreak strains/clones within epidemic clones (8). Since the middle of the 1990s, the CRISPR locus of *Mycobacterium tuberculosis* has been studied extensively, and the high degree of polymorphism of the spacer content has led to the development of a subtyping method known as spoligotyping (9). Several studies have reported the presence of two CRISPR loci in *Salmonella* (10, 11), and CRISPR polymorphisms are strongly correlated with serovars and subtypes (12). Recently, three studies suggested that CRISPR loci may provide information useful for typing and subtyping of *Salmonella* (8, 13, 14), and they presented a CRISPR typing method (hereinafter referred to as conventional

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TABLE 1 List of 82 *Salmonella* isolates that were analyzed in this study

Strain	Serovar	Origin	Source	Yr of isolation	Serotype (O:H1:H2)
BJ0047	Agona	Beijing	Human	2009	1,4,12:f,g,s:−
BJ0048	Agona	Beijing	Human	2009	1,4,13:f,g,s:−
NJ92234	Agona	Nanjing	Human	2011	1,4,15:f,g,s:−
SY628	Agona	Shenyang	Human	2010	1,4,14:f,g,s:−
B3V3-2	Albany	Xinjiang	Human	2009	8,20:Z4,Z24:−
NJ81658-1	Choleraesuis	Nanjing	Human	2011	6,7:C:1,5
NJ81658-2	Choleraesuis	Nanjing	Human	2011	6,7:C:1,5
BJ0053	Derby	Beijing	Human	2009	1,4,5,12:f,g:1,2
SY454	Derby	Shenyang	Human	2010	1,4,5,12:f,g:1,2
B43V2	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
B63V1	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
BJ0046	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
BJ0049	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
BJ0051	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
BJ0065	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
BJ0068	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
BJ0069	Enteritidis	Beijing	Human	2009	1,9,12:g,m:1,7
C3V1	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
E19V2	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
GZ0010	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0013	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0015	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0020	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0023	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0027	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
GZ0042	Enteritidis	Guangzhou	Human	2009	1,9,12:g,m:1,7
NJ81625	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ82854	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92143	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92341-1	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92341-2	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92374	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92432	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
NJ92663	Enteritidis	Nanjing	Human	2011	1,9,12:g,m:1,7
SY020	Enteritidis	Shenyang	Human	2009	1,9,12:g,m:1,7
SY070	Enteritidis	Shenyang	Human	2009	1,9,12:g,m:1,7
SY204	Enteritidis	Shenyang	Human	2010	1,9,12:g,m:1,7
SY330	Enteritidis	Shenyang	Human	2010	1,9,12:g,m:1,7
SY609	Enteritidis	Shenyang	Human	2010	1,9,12:g,m:1,7
SY637	Enteritidis	Shenyang	Human	2010	1,9,12:g,m:1,7
XJ001	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
XJ002	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
XJ003	Enteritidis	Xinjiang	Human	2009	1,9,12:g,m:1,7
NJ92713	Heidelberg	Nanjing	Human	2011	1,4,15,12:r:1,2
SY588-3	Indiana	Shenyang	Human	2010	1,4,12:Z:1,7
JN0005	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0011	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0014	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0015	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0016	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0025	Infantis	Jinan	Human	2009	6,7:r:1,5
JN008	Infantis	Jinan	Human	2009	6,7:r:1,5
JN009	Infantis	Jinan	Human	2009	6,7:r:1,5
JN0096	Infantis	Jinan	Human	2009	6,7:r:1,5
BJ0055	Kentucky	Beijing	Human	2009	8,20:i:Z6
BJ0058	Kentucky	Beijing	Human	2009	8,20:i:Z6
BJ0059	Kentucky	Beijing	Human	2009	8,20:i:Z6
BJ0060	Kentucky	Beijing	Human	2009	8,20:i:Z6
BJ0061	Kentucky	Beijing	Human	2009	8,20:i:Z6
BJ0063	Lexington	Beijing	Human	2009	6,7:f,g:−
GZ001	Litchfield	Guangzhou	Human	2009	6,8:l,v:1,2
NJ85022	Litchfield	Nanjing	Human	2011	6,8:l,v:1,2
NJ91889	Montevideo	Nanjing	Human	2011	6,7:g,m,p,s:1,2,7
JN0010	Newport	Jinan	Human	2009	6,8:e,h:1,2
BJ0054	Newwington	Beijing	Human	2009	3,15:e,h:1,6
BJ0062	Newwington	Beijing	Human	2009	3,15:e,h:1,6
NJ92291	Paratyphi a	Nanjing	Human	2011	1,21,2:a:1,5
BJ0064	Paratyphi b	Beijing	Human	2009	1,4,5,12:b:1,2
BJ0066	Paratyphi b	Beijing	Human	2009	1,4,5,12:b:1,2
BJ0067	Paratyphi b	Beijing	Human	2009	1,4,5,12:b:1,2
GZ0012	Paratyphi b	Guangzhou	Human	2009	1,4,5,12:b:1,2
GZ0036	Paratyphi b	Guangzhou	Human	2009	1,4,5,12:b:1,2
GZ0041	Paratyphi b	Guangzhou	Human	2009	1,4,5,12:b:1,2
XJ004	Paratyphi b	Xinjiang	Human	2009	1,4,5,12:b:1,2
BJ0052	Senftenberg	Beijing	Human	2009	1,3,19:g,s,t:−
NJ92176	Senftenberg	Nanjing	Human	2011	1,3,19:g,s,t:−
GZ0045	Stanley	Guangzhou	Human	2009	1,4,5,12,27:d:1,2
NJ92671	Thompson	Nanjing	Human	2011	6,7,k,1,5
XJ005	Typhi	Xinjiang	Human	2009	9,12,Vi:d:−
BJ302	Typhimurium	Beijing	Human	2010	1,4,5,12:i:1,2
NJ81674	Typhimurium	Nanjing	Human	2011	1,4,5,12:i:1,2
NJ85592	Typhimurium	Nanjing	Human	2011	1,4,5,12:i:1,2

CRISPR typing [CCT]) that is based on all spacers in both CRISPR loci. However, only a limited number of serotypes from a single geographic area were studied using the CCT method because it is complicated and labor intensive.

The functional studies of the CRISPR loci suggested that, upon infection with a foreign element, part of the *Salmonella* genome is typically incorporated into the leader end of the CRISPR array as a spacer and the repeat is duplicated (15). Therefore, these spacers are integrated into the CRISPR locus in a polarized manner and the newly incorporated spacers adjoin the leader array in the CRISPR locus (7). Therefore, we think the newly incorporated spacer adjoining the leader array may be an effective molecular marker for subtyping of *Salmonella* isolates. We evaluated these assumptions using CRISPR databases, and we propose a new method for *Salmonella* typing and subtyping, called CRISPR locus spacer pair typing (CLSPT), in which only the newly incorporated spacers in two CRISPR loci are analyzed.

MATERIALS AND METHODS

Bacterial isolates. Eighty-two *Salmonella* isolates were separated from 4,901 fecal samples obtained during January 2009 to March 2011 from patients with diarrhea or dysentery at hospitals in 6 provinces within the following regions of China: Beijing, Jiangsu, Guangdong, Shandong, Liaoning, and Xinjiang provinces (Table 1). Immunological serotyping was completed using diagnostic sera (SSI Diagnostica, Hillerød, Denmark) for *Salmonella* according to the manufacturer's instructions. The collection of *Salmonella enterica* serotypes was comprised of 34 strains of *S.* Enteritidis, 9 strains of *S.* Infantis, 7 strains of *S.* Paratyphi B, 5 strains of *S.* Kentucky, 4 strains of *S.* Agona, 3 strains of *S.* Typhimurium, and other common epidemic serotypes (Table 1). All isolates were stored at −80°C in 20% glycerol. When necessary, some isolates were grown in Luria-Bertani broth at 37°C overnight. Total DNA was extracted using the TIANamp bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions and stored at −20°C before use.

To evaluate our method *in silico*, we obtained CRISPR arrays from 537 Salmonella isolates in the Pasteur Institute's CRISPR Database (http://www.pasteur.fr/recherche/genopole/PF8/crispr/CRISPRDB.html) which comprised 131 strains of *S.* Enteritidis, 102 strains of *S.* Typhimurium, 28 strains of *S.* Paratyphi B, 15 strains of *S.* Newport, 15 strains of *S.* Typhi, 13 strains of *S.* Kentucky, 11 strains of *S.* Agona, and other common epidemic serotypes. These serotypes belonged to the two species of the *Salmonella* genus, *S. enterica* and *S. bongori*, and the six *S. enterica* subspecies, *S. enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica*, and *houtenae*.

PCR amplification and sequencing of the CRISPR loci. We amplified the CRISPR 1 locus with forward primer A1 (5′-GTRGTRCGGATAATG CTGCC-3′) and reverse primer A2 (5′-CGTATTCCGGTAGATBTDGA TGG-3′). To amplify the CRISPR 2 locus, we used forward primer B1 (5′-GAGCAATACYYTRATCGTTAACGCC-3′) and reverse primer B2 (5′-GTTGCDATAKGTYGRTRGRATGTRG-3′). All the primers were

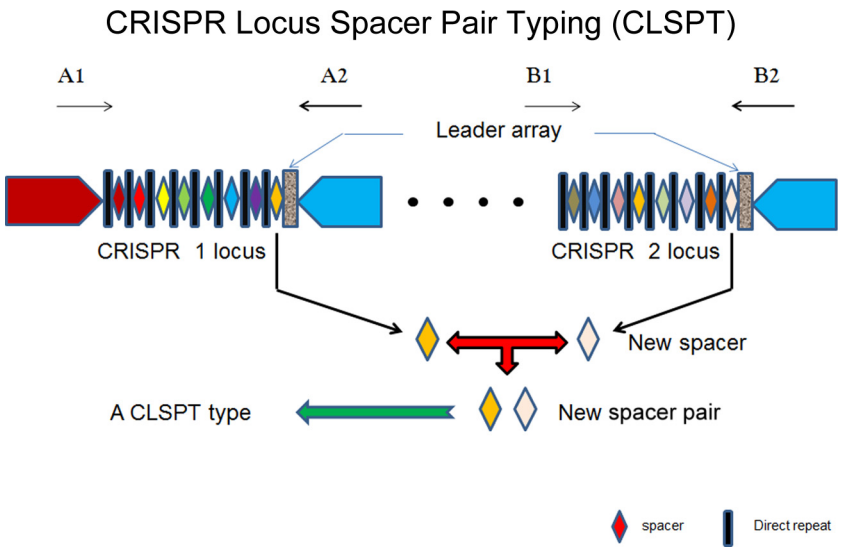


FIG 1 The new method is based only on the newly incorporated spacers adjoining the leader array in both CRISPR loci. We used the spacer adjoining the leader array in each CRISPR locus to form a spacer pair to represent each isolate. Spacers and direct repeats were visualized as described by L. Fabre (14) et al.

designed by L. Fabre (14) and synthesized by Sangon Biotech (Shanghai, China).

A 50-μl PCR mixture volume contained 0.25 μl TaKaRa *Ex Taq* DNA polymerase, 5 μl 10× *Ex Taq* buffer (Mg^{2+} plus), 4 μl deoxynucleoside triphosphate (dNTP) mix (2.5 mM each), 5 μl DNA template, 2 μl each forward primer and reverse primer (final concentration, 0.2 μM), and 31.75 μl sterile double-distilled water. The cycling conditions were as follows: 10 min at 94°C for denaturation (1 cycle), followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for polymerization, followed by an additional 10 min at 72°C for extension. The PCR products were sequenced with a BigDye Terminator kit, version 3.1 (Sangon Biotech, Shanghai, China), using an ABI 3730XL apparatus.

CLSPT. In CLSPT, we performed sequencing with the corresponding reverse primer and identified the newly incorporated spacers, which are located between the first and second direct repeats (DRs) adjoining the leader array. The CLSPT profiles are the sequences which are composed of the two newly incorporated spacers from the CRISPR 1 and CRISPR 2 loci. In order to predict their serovars, we also assigned each CLSPT profile a unique type composed of the two newly incorporated spacers' names (Fig. 1). The CLSPT profiles were clustered with the BioNumerics software (Applied Maths, Austin, TX, USA) using a categorical coefficient and a graphing method called the minimum spanning tree.

CCT. The sequences of direct repeats and spacers in CRISPR 1 and CRISPR 2 were identified by using CRISPRfinder (http://crispr.u-psud.fr/Server/). All sequences from this study were submitted as a batch to a private database in CRISPRdb (http://crispr.u-psud.fr/CRISPRcompar/private/PrivateDatabase.php) under accession numbers 403_15795 to 403_15895. The analyses of the spacer arrangements were performed using CRISPRcompar (6). Different allelic types (ATs; sequences with at least a 1-nucleotide difference, or a 1-spacer difference in the case of CRISPRs) were assigned arbitrary numbers. The combination of 2 alleles (CRISPR 1 and CRISPR 2) determined the allelic profile, and each unique allelic profile was designated a unique CCT type.

MLST. MLST was carried out using the protocols described on the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica/documents/primersEnterica_html). The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. PCR amplicons were sequenced at Sangon Biotech (Shanghai Biotech, China). Sequences were assembled and analyzed using Lasergene 7.1 software (DNAStar). Sequence type (ST) numbers were assigned by submitting the sequences to the *Salmonella* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

PFGE. Restriction endonuclease digestion was carried out using XbaI (TaKaRa, Dalian, China) at 37°C for 3 h. DNA macrorestriction fragments were resolved on 1% SeaKem Gold agarose (Lonza, Rockland, ME, USA) using a CHEF Mapper PFGE system for over 19 h. *S. enterica* serovar Braenderup strain H9812 was used as the reference strain (16). BioNumerics version 6.0 was used to analyze the PFGE patterns. Similarity analysis was performed using the Dice coefficient, and clustering was performed using the unweighted-pair group method by arithmetic mean with a 1.5% tolerance limit.

RESULTS

CLSPT. For 82 *Salmonella* isolates, both CRISPR 1 and CRISPR 2 loci were identified. In total, there are 2,998 spacers in both CRISPR loci. The newly incorporated spacer adjoining the leader array in the CRISPR 1 locus has 23 different alleles, by which 77 of the 82 (93%) isolates' serotypes could be correctly predicted. The isolates that could not be predicted included *S.* Paratyphi B BJ0066, *S.* Typhimurium NJ85436, *S.* Newport JN0010, *S.* Newington BJ0062, and *S.* Paratyphi B BJ0064. In addition, there were 25 different newly incorporated spacer alleles in the CRISPR 2 locus, by which 78 of the 82 (95%) isolates' serotypes were correctly predicted. The isolates that could not be predicted included *S.* Choleraesuis NJ81658-1, *S.* Montevideo NJ91889, *S.* Albany XJB3V3-2, and *S.* Senftenberg NJ92176. We obtained 30 different alleles and predicted 82 of 82 (100%) serotypes correctly when the combination of the two newly incorporated spacers was considered (Table 2). Therefore, 82 *Salmonella enterica* strains were subtyped into 30 different CRISPR locus spacer pair types (CLSPTs) and all of their serotypes were clearly separated. In addition, the minimum spanning tree constructed based on CLSPTs in BioNumerics (Fig. 2) also supported the fact that the CLSPTs are highly correlated with the serotypes.

High consistency of typing results between CLSPT and traditional serotyping. To evaluate the consistency between the results of CLSPT and those of the traditional serotyping method, 537 *Salmonella* strains belonging to 101 serovars in the Pasteur

TABLE 2 CRISPR locus spacer pair types, conventional CRISPR types, multilocus sequence types, and pulsed-field gel electrophoresis types of the 82 *Salmonella* isolates[a]

Strain	*Salmonella* serotype	ST	PT	CLSPT[b]	CCT
NJ92234	Agona	13	PT36	Ago13 AgoB8	1
BJ0047	Agona	13	PT59	Ago13 AgoB8	1
BJ0048	Agona	13	PT59	Ago13 AgoB8	1
SY628	Agona	13	PT59	Ago13 AgoB8	1
B3V3-2	Albany	292	PT65	Abl4* AlbB17	2
NJ81658-1	Choleraesuis	145	PT33	Chol3 CholB6	3
NJ81658-2	Choleraesuis	145	PT58	Chol3 MonB54	4
SY454	Derby	40	PT76	Der21 DerB11	6
BJ0053	Derby	40	PT77	Der21 DerB12	5
SY609	Enteritidis	11	PT37	Ent8 EntB9	8
NJ82854	Enteritidis	11	PT82	Ent8 EntB9	7
BJ0049	Enteritidis	11	PT84	Ent8 EntB9	7
BJ0051	Enteritidis	11	PT84	Ent8 EntB9	7
BJ0069	Enteritidis	11	PT84	Ent8 EntB9	7
E19V2	Enteritidis	11	PT84	Ent8 EntB9	7
GZ0010	Enteritidis	11	PT84	Ent8 EntB9	7
GZ0015	Enteritidis	11	PT84	Ent8 EntB9	7
GZ0042	Enteritidis	11	PT84	Ent8 EntB9	7
NJ92143	Enteritidis	11	PT84	Ent8 EntB9	7
NJ92341-1	Enteritidis	11	PT84	Ent8 EntB9	7
NJ92341-2	Enteritidis	11	PT84	Ent8 EntB9	7
SY020	Enteritidis	11	PT84	Ent8 EntB9	7
SY070	Enteritidis	11	PT84	Ent8 EntB9	7
SY204	Enteritidis	11	PT84	Ent8 EntB9	7
SY330	Enteritidis	11	PT84	Ent8 EntB9	8
SY637	Enteritidis	11	PT84	Ent8 EntB9	8
XJ001	Enteritidis	11	PT84	Ent8 EntB9	9
XJ002	Enteritidis	11	PT84	Ent8 EntB9	10
XJ003	Enteritidis	11	PT84	Ent8 EntB9	10
B63V1	Enteritidis	11	PT85	Ent8 EntB9	7
C3V1	Enteritidis	11	PT85	Ent8 EntB9	7
NJ92374	Enteritidis	11	PT87	Ent8 EntB9	7
NJ92663	Enteritidis	11	PT87	Ent8 EntB9	7
BJ0065	Enteritidis	11	PT88	Ent8 EntB9	7
GZ0020	Enteritidis	11	PT88	Ent8 EntB9	7
GZ0023	Enteritidis	11	PT88	Ent8 EntB9	7
BJ0046	Enteritidis	11	PT91	Ent8 EntB9	7
BJ0068	Enteritidis	11	PT92	Ent8 EntB9	7
B43V2	Enteritidis	11	PT93	Ent8 EntB9	7
GZ0013	Enteritidis	11	PT93	Ent8 EntB9	7
NJ81625	Enteritidis	11	PT95	Ent8 EntB9	7
NJ92432	Enteritidis	11	PT97	Ent8 EntB9	7
GZ0027	Enteritidis	11	PT98	Ent8 EntB9	7
NJ92713	Heidelberg	15	PT45	Heid14* STMB24	11
SY588-3	Indiana	17	PT31	Ind13 IndB13	12
JN0005	Infantis	32	PT05	Inf34* InfB26	13
JN0011	Infantis	32	PT05	Inf31 InfB26	14
JN0014	Infantis	32	PT05	Inf31 InfB26	14
JN0015	Infantis	32	PT05	Inf31 InfB26	14
JN0016	Infantis	32	PT05	Inf31 InfB26	14
JN0025	Infantis	32	PT05	Inf31 InfB26	14
JN008	Infantis	32	PT09	Inf31 InfB26	14
JN009	Infantis	32	PT09	Inf31 InfB26	14
JN0096	Infantis	32	PT09	Inf31 InfB26	14
BJ0055	Kentucky	198	PT78	Ken26 KenB41	15
BJ0058	Kentucky	198	PT78	Ken26 KenB41	15
BJ0059	Kentucky	198	PT78	Ken26 KenB41	15
BJ0060	Kentucky	198	PT78	Ken26 KenB41	15
BJ0061	Kentucky	198	PT78	Ken26 KenB41	15
BJ0063	Lexington	247	PT74	Lex16 LexB6	16
GZ001	Litchfield	1499	PT24	Bovis13 BovB10var1	17
NJ85022	Litchfield	214	PT28	Lit1 LitB1	18
NJ91889	Montevideo	4	PT14	Mon43 CholB6	19
BJ0054	Newington	516	PT71	Der26 NewB1*	21
BJ0062	Newington	516	PT71	Inf34* NewB1*	22
JN0010	Newport	33	PT11	Inf34* HadB30	20
NJ92291	Paratyphi A	85	PT75	ParA5 ParAB2	23
BJ0064	Paratyphi B	34	PT39	Inf34* STMB33	24
BJ0066	Paratyphi B	34	PT39	STM6 STM31	25
BJ0067	Paratyphi B	34	PT39	STM6 STM31	25
GZ0041	Paratyphi B	34	PT39	STM6 STM31	25
GZ0012	Paratyphi B	34	PT41	STM6 STM31	25
XJ004	Paratyphi B	328	PT42	STM6 STM24	26
GZ0036	Paratyphi B	34	PT43	STM6 STM31	25
NJ92176	Senftenberg	14	PT01	Sen16 AlbB17	28
BJ0052	Senftenberg	14	PT13	Sen16 SenB25	27
GZ0045	Stanley	29	PT17	Sta3 StaB5	29
NJ92671	Thompson	26	PT23	Tho15 ThoB7	30
XJ005	Typhi	2	PT62	Typhi6 AnaB14	31
BJ302	Typhimurium	19	PT47	STM24 STMB31	32
NJ81674	Typhimurium	19	PT52	STM6 STMB31	33
NJ85592	Typhimurium	19	PT54	STM6 STMB31	33

[a] CLSPT, CRISPR locus spacer pair type; CCT, conventional CRISPR type; ST, multilocus sequence type; PT, PFGE pulsotype.
[b] *, novel spacer (not found in the Pasteur Institute's CRISPR database) identified in the current study. We used the spacer nomenclature described by L. Fabre (14). The three- or four-letter prefix in the spacer name indicates the serotype from which the spacer was first extracted, the suffix B indicates that the spacer was found in the CRISPR 2 locus, and spacers were numbered consecutively in order of discovery. Abl4*, CACATGTCAGATGTTATTTCCAAGGCGGAGC; Heid14*, ACCGTTACGCGCATCTTGAAAATCAGCTTCGA; Inf34*, GTTCCGTACTGCAGTGGTGAATTATCAGTAAT; NewB1*, TTAGTATTTGTAGCGGTTTTCCGGAGAAAACG.

Institute's CRISPR Database were selected. Ninety-three different alleles were identified using CLSPT. Then, we established a CLSPT/serotype dictionary (see Table S1 in the supplemental material) by which the serovars of 514 of 537 (95%) strains were correctly correlated to the CLSPTs. In general, one or several CLSPTs are specific to one serovar. For example, the CLSPT of 13/15 *S.* Typhi strains is Typhi6 EntB0var1, while 2/15 are Typhi3 EntB0var1. However, nine CLSPTs corresponded to several serotypes, indicating that isolates of the different serotypes might have been separated from the same phage/plasmid pool, infected with the same phage, or had the same evolutionary origin. In general, a majority of the CLSPTs are specific to corresponding serotypes, and the addition of an extra spacer in the CRISPR locus is an alternative way for the ambiguous results to occur.

In order to check the consistency of the results of CLSPT and serotyping, we proposed to use the most likely serovar to resolve these ambiguous results. For example, 121 isolates had the same CLSPT, Ent8 EntB9, including 115 isolates of *S.* Enteritidis, 3 isolates of *S. enterica* Nitra, 2 of *S. enterica* Rosenberg, and 1 of *S. enterica* Blegdam. Thus, *S.* Enteritidis was the most likely predicted serotype. The kappa coefficient demonstrated a kappa of 0.9872, the 95% confidence interval (CI) was 0.9771 to 0.9974, and the Matthew's correlation coefficient (MCC) was 0.9712.

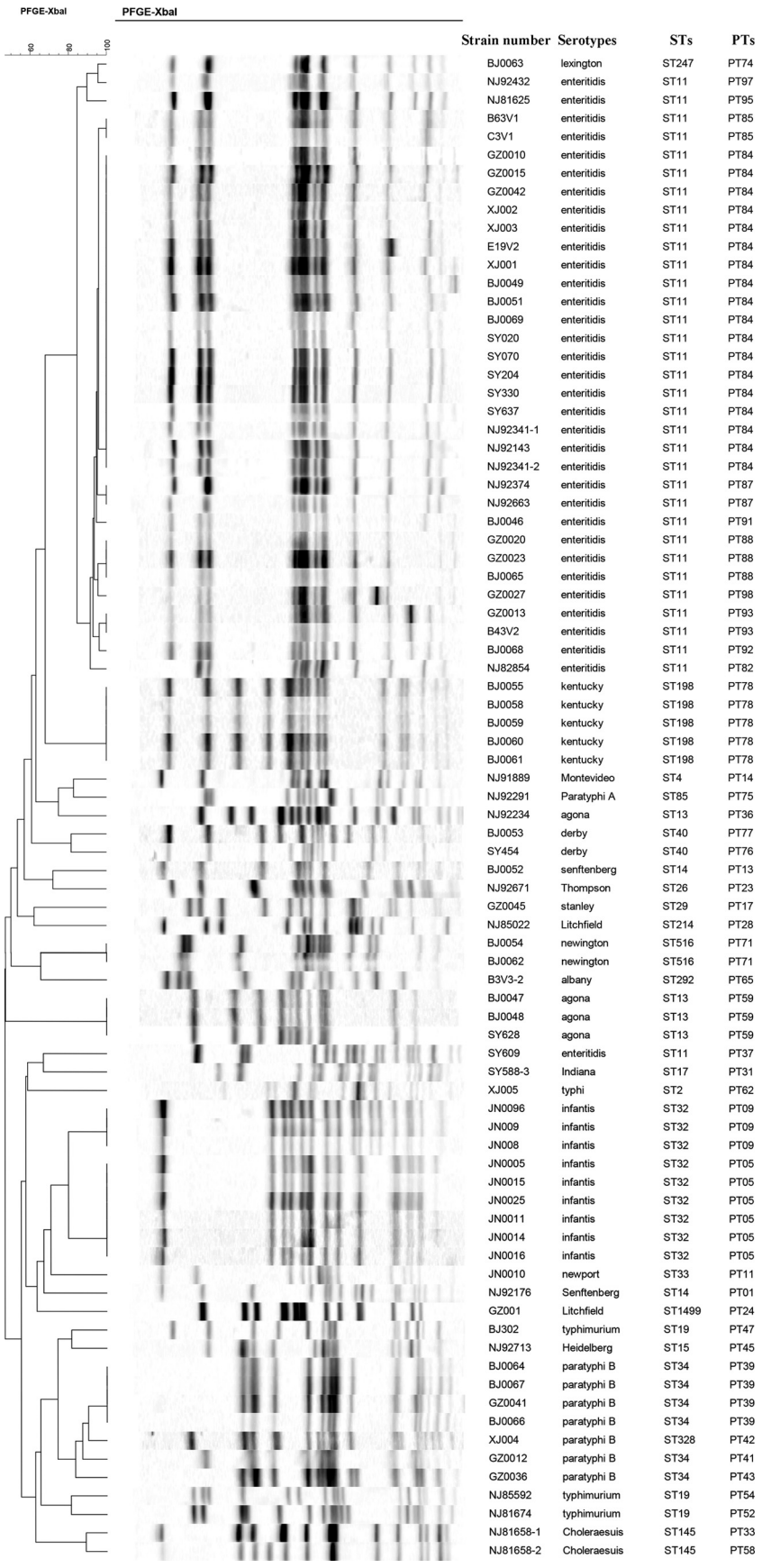
Comparison of discriminatory power between CLSPT, CCT, MLST, and PFGE. By MLST, we divided the 82 *Salmonella* isolates

FIG 2 A minimum spanning tree has been constructed based on CLSPTs using the strains listed in Table 2. In the tree, the corresponding serotypes are circled. CLSPT types are represented by circles, and the size of a circle indicates the number of strains with this particular type. There is no ambiguous result such as one CLSPT type corresponding to two or more serotypes. The halos surrounding the various types denote the groupings obtained by Bionumerics analysis, which indicate that they may be separated from the related phage/plasmid pool. A minimum neighbor difference of 1 was used for the creation of groups.

belonging to 21 serovars into 23 STs (see Table S2 in the supplemental material). Most STs ($n = 21$, 91.3%) were completely consistent with their serovars. Two of the *S.* Litchfield isolates were divided into 2 of the STs (ST214 and ST1499), and the *S.* Paratyphi B isolates were divided into 2 of the STs, ST34 ($n = 6$; 85.7%) and ST328 ($n = 1$; 14.3%). By CLSPT, we subtyped the 82 *Salmonella* isolates into 30 CLSPTs. The most frequent CLSPT type was Ent8 EntB9 ($n = 34$, 41.5%), and we were able to further subtype isolates of ST145, ST40, ST32, ST516, ST34, ST14, and ST19 into 14 different CLSPT types (Table 2), while by using conventional CRISPR typing, we subtyped the 82 *Salmonella* isolates into 33 CCTs by analyzing all the spacers contained in two CRISPR loci. The most frequent CCT was CCT7 ($n = 28$, 34.1%), and we subtyped isolates ST145, ST11, ST40, ST32, ST516, ST34, ST14, and ST19 into 18 different CCTs (Table 2). Using PFGE, we identified 43 profiles among the 82 isolates (Fig. 3), and the most frequent PFGE pulsotype (PT) was PT84 ($n = 17$, 21%). In addition, we subtyped 10 STs (ST145, ST11, ST13, ST198, ST40, ST32, ST516, ST34, ST14, and ST19) into 30 different PTs. In the 82 *Salmonella* isolates, the discriminatory power (discriminatory index [DI]) of CLSPT was 0.8145. This means that there should be an 81% probability that two unrelated isolates can be separated using the CLSPT scheme. The discriminatory powers of MLST, CCT, and PFGE were 0.8088, 0.8684, and 0.9455, respectively, among these isolates.

DISCUSSION

Several molecular subtyping methods have been developed for studying the epidemiology of *Salmonella*, including PFGE and MLST (17). MLST has commonly been used in the subtyping of bacteria (18), and PFGE is currently the gold standard method used by public health surveillance laboratories for tracking foodborne pathogens (17), but both of them still have some disadvantages. Two studies recently suggested that CRISPR loci might provide information useful for typing (8, 13). With the use of the CRISPR typing method in *Salmonella*, more strategies have emerged. For example, (i) variations in the number and type of spacers can be used to track strains (14), (ii) CRISPOL, for CRISPR polymorphism, a bead-based liquid hybridization assay, is a high-throughput method for subtyping a serotype or a monophasic variant in real time (14), and (iii) a novel MLST scheme, CRISPR-multi-virulence-locus sequence typing (MVLST), using the virulence genes *sseL* and *fimH* and CRISPRs, is even better than PFGE in discrimination (14).



Strain number	Serotypes	STs	PTs
BJ0063	lexington	ST247	PT74
NJ92432	enteritidis	ST11	PT97
NJ81625	enteritidis	ST11	PT95
B63V1	enteritidis	ST11	PT85
C3V1	enteritidis	ST11	PT85
GZ0010	enteritidis	ST11	PT84
GZ0015	enteritidis	ST11	PT84
GZ0042	enteritidis	ST11	PT84
XJ002	enteritidis	ST11	PT84
XJ003	enteritidis	ST11	PT84
E19V2	enteritidis	ST11	PT84
XJ001	enteritidis	ST11	PT84
BJ0049	enteritidis	ST11	PT84
BJ0051	enteritidis	ST11	PT84
BJ0069	enteritidis	ST11	PT84
SY020	enteritidis	ST11	PT84
SY070	enteritidis	ST11	PT84
SY204	enteritidis	ST11	PT84
SY330	enteritidis	ST11	PT84
SY637	enteritidis	ST11	PT84
NJ92341-1	enteritidis	ST11	PT84
NJ92143	enteritidis	ST11	PT84
NJ92341-2	enteritidis	ST11	PT84
NJ92374	enteritidis	ST11	PT87
NJ92663	enteritidis	ST11	PT87
BJ0046	enteritidis	ST11	PT91
GZ0020	enteritidis	ST11	PT88
GZ0023	enteritidis	ST11	PT88
BJ0065	enteritidis	ST11	PT88
GZ0027	enteritidis	ST11	PT98
GZ0013	enteritidis	ST11	PT93
B43V2	enteritidis	ST11	PT93
BJ0068	enteritidis	ST11	PT92
NJ82854	enteritidis	ST11	PT82
BJ0055	kentucky	ST198	PT78
BJ0058	kentucky	ST198	PT78
BJ0059	kentucky	ST198	PT78
BJ0060	kentucky	ST198	PT78
BJ0061	kentucky	ST198	PT78
NJ91889	Montevideo	ST4	PT14
NJ92291	Paratyphi A	ST85	PT75
NJ92234	agona	ST13	PT36
BJ0053	derby	ST40	PT77
SY454	derby	ST40	PT76
BJ0052	senftenberg	ST14	PT13
NJ92671	Thompson	ST26	PT23
GZ0045	stanley	ST29	PT17
NJ85022	Litchfield	ST214	PT28
BJ0054	newington	ST516	PT71
BJ0062	newington	ST516	PT71
B3V3-2	albany	ST292	PT65
BJ0047	agona	ST13	PT59
BJ0048	agona	ST13	PT59
SY628	agona	ST13	PT59
SY609	enteritidis	ST11	PT37
SY588-3	Indiana	ST17	PT31
XJ005	typhi	ST2	PT62
JN0096	infantis	ST32	PT09
JN009	infantis	ST32	PT09
JN008	infantis	ST32	PT09
JN0005	infantis	ST32	PT05
JN0015	infantis	ST32	PT05
JN0025	infantis	ST32	PT05
JN0011	infantis	ST32	PT05
JN0014	infantis	ST32	PT05
JN0016	infantis	ST32	PT05
JN0010	newport	ST33	PT11
NJ92176	Senftenberg	ST14	PT01
GZ001	Litchfield	ST1499	PT24
BJ302	typhimurium	ST19	PT47
NJ92713	Heidelberg	ST15	PT45
BJ0064	paratyphi B	ST34	PT39
BJ0067	paratyphi B	ST34	PT39
GZ0041	paratyphi B	ST34	PT39
BJ0066	paratyphi B	ST34	PT39
XJ004	paratyphi B	ST328	PT42
GZ0012	paratyphi B	ST34	PT41
GZ0036	paratyphi B	ST34	PT43
NJ85592	typhimurium	ST19	PT54
NJ81674	typhimurium	ST19	PT52
NJ81658-1	Choleraesuis	ST145	PT33
NJ81658-2	Choleraesuis	ST145	PT58

FIG 3 PFGE dendrogram of 82 *Salmonella* strains, with strain number, serotype, ST, and PT for each strain.

In this study, we proposed a new method, CRISPR locus spacer pair typing (CLSPT), to type and subtype *Salmonella* isolates. This new method only needs three steps. First, PCR is used to amplify both CRISPR loci. Second, the newly incorporated spacer in both CRISPR loci is investigated by reverse sequencing of the PCR products using reverse primers. Third, the sequenced newly incorporated spacers in CRISPR 1 and CRISPR 2 are used to form a pair to represent a new CRISPR type, and the explicit serovar is identified with the CLSPT/serotype dictionary (see Table S1 in the supplemental material). Thus, the CLSPT method makes the CRISPR typing method for *Salmonella* simpler and CRISPOL (14) more realizable in all *Salmonella* bacteria. Meanwhile, our study indicated that (i) CLSPT has a considerable discriminatory power (DI = 0.8145) and may provide an ideal balance between a high discriminatory power and a convenient process, (ii) CLSPT results have a high level of consistency (kappa = 0.9872, MCC = 0.9712) with the results of traditional serotyping, such that CLSPT may become a new procedure in *Salmonella* serovar prediction, and (iii) CLSPT may be the least expensive method for typing and subtyping of *Salmonella*. Traditional serotyping costs $35 to $185 per isolate (19), and the cost of 7-gene MLST is about $35 per isolate (20), whereas CLSPT was predicted to only cost about $5 to $10 per isolate. Besides the excellent time savings, low cost, high throughput, and considerable discriminatory power, CLSPT as a typing and subtyping method in *Salmonella* also contains geographic information, which other methods do not. A previous study demonstrated that bacteria from distant geographic locations had strikingly different spacer arrangements, possibly due to the existence of unique phage/plasmid pools in these different geographic locations (21). However, we speculate that newly incorporated spacers may represent unique ecotypes that are distinct from STs and serovars. Of note, an acquisition of a newly incorporated spacer in response to phage and/or plasmids has not yet been reported for *Salmonella*.

The results in this study are very preliminary, and further study is necessary to enlarge the relatively small number of *S.* Enteritidis isolates causing infection and to test isolates that are not *S. enterica* subsp. *enterica*. Second, it is imperative that we enlarge our CLSPT/serotype dictionary, since some common serovars, such as *S.* Newington, have not yet been recorded in this dictionary. It would be ideal for this dictionary to include a majority of the >2,600 *Salmonella* serovars or at least a majority of the serovars that are typically encountered. As studies about CRISPRs move along, we believe that the typing and subtyping methods based on CRISPRs will become more critical for *Salmonella* characterization and that this new CLSPT method will provide considerable advantages over other molecular serotyping methods. Particularly because this new method is simple and rapid and its results have high accordance with serotyping, it can become a valuable epidemiologic tool and may be widely used in laboratory surveillance of *Salmonella* infections. For example, it may be especially useful and time saving to predict the serovars of unknown isolates using CLSPT before doing the traditional serotyping. Also, the method is feasible for more laboratories or even primary units of disease control without well trained laboratory staff or sophisticated typing equipment, such as PFGE systems. Thus, the CLSPT method could greatly improve the efficiency and scope of epidemiological investigations.

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