Diversity of CRISPR Array and *In silico* PCR-RFLP Typing Application in *Salmonella enterica*

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Abstract

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is the special arrangements of the nucleotide array with alternating between short direct repeat separated by a short spacer sequence. Along with the CRISPR system, they encode an adaptive immune system of bacteria that protects bacterial cell against bacteriophage and plasmid infection. The CRISPR system is found diversely among bacterial species; moreover, these arrays are able to transfer from generation to generation via vertical transfer. However, reports regarding the survey of the CRISPR arrays diversity in *Salmonella enterica* isolated from various sources, in order to further contribute to the understanding and application, is still limited in Thailand. Here we described CRISPR-I and CRISPR-II systems found in *S. enterica*, following the analysis of 117 isolates of *S. enterica* from various sources. The results found that 48 (41%) and 71 (83%) of *S. enterica* isolates were positive for CRISPR-I and CRISPR-II, respectively. The nucleotide analysis showed that the CRISPR-I and CRISPR-II spacers varied between each isolate, ranged between 2- 15 and 1- 17 spacers, respectively. Moreover, sequencing analysis indicated that the direct repeat region in both CRISPR-I and CRISPR-II has a high diversity. Our study also showed that *in silico* PCR-RFLP of CRISPR region, and phylogenetic tree construction of the spacers from *Salmonella enterica* serovar Weltevreden (S. Weltevreden) isolated from different sources indicated that it has discrimination ability between intra-serovar. These results imply that the CRISPR array could probably apply as a molecular marker for epidemiological study in *S. enterica*.

Keywords: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR); diversity; *Salmonella enterica*; *In silico* PCR-RFLP

INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are a special characteristic of a DNA arrays containing conserved short repetitive direct repeat, which are embedded between a short variable sequence called spacer (Rath *et al.*, 2015). The nucleotide sequence of spacers is similar to some parts of those derived from invading bacteriophage or plasmid, suggesting that the CRISPR arrays are part of a specific immune system against previous infectious bacteriophage and plasmid (Horvath *et al.*, 2010; Terns *et al.*, 2011; van der Oost *et al.*, 2009). It is widely acknowledged that bacteria have a mechanism to uptake and integrate a new spacer DNA from bacteriophage or plasmid into their genome (Barrangou *et al.*, 2007; Horvath *et al.*, 2010). After the acquisition of spacers, the direct repeat and spacers are transcribed as a short RNA called CRISPR-RNA (crRNA). This crRNA coupled together with a CRISPR-associated protein (Cas) subsequently cleave known bacteriophage or plasmids DNA (Marraffini *et al.*, 2010; Pougacha *et al.*, 2012). Diversity in spacer number and nucleotide sequences have been report even in the same bacterial species. This probably due to the differences between the habitat, hosts, as well as loss or duplication of the acquired CRISPR arrays, which resulted in very extensive diversity that occur both inter-species and intra-species (Fabre *et al.*, 2012; Horvath *et al.*, 2008; Koonin *et al.*, 2013; Pourcel *et al.*, 2005; Sheludchenko *et al.*, 2015). Moreover, these variation arrays are able to be transferred from the parent strain to the daughter strain via vertical transfer (Iranzo *et al.*, 2013).

According to the characters as mentioned above, the CRISPR arrays have diverse aspect for applications in biomedical science. For example, the use of CRISPR arrays as genetic markers in the epidemiological study, especially for outbreak investigations

which have been proved to be efficient in several pathogenic bacteria. It was first described in *Mycobacterium tuberculosis* complex (MTBC), which indicated that each different strain of MTCB contains polymorphisms in the CRISPR arrays (Groenen *et al.*, 1993; Kamerbeek *et al.*, 1997). A comparative study of genotyping for outbreak tracking of *Campylobacter jejuni* by CRISPR sequencing, amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) demonstrated that the power of discrimination of those three techniques was closely related (Schouls *et al.*, 2003). The high resolution discrimination by CRISPR genotyping was also shown in *Yersinia pestis* (Pourcel *et al.*, 2004; Pourcel *et al.*, 2005).

In the case of *S. enterica*, an enteric pathogen commonly causes of foodborne illness in human, it has tremendous diversity in the subspecies and serovar levels since it comprises of six subspecies and more than 2,500 serovars (Kauffmann, 1971). Considering this, an efficient laboratory method for serotyping is very important for epidemiological study. Pulsed-field gel electrophoresis (PFGE), the gold standard method developed for separation of large bacterial chromosome, has proven helpful in determining the relationship of serotype for tracking an outbreak of *S. enterica* (Zou *et al.*, 2010; Zou *et al.*, 2012). However, PFGE is a complicated technique that requires skilled personnel and needs an expensive instrument. Comparative study of CRISPR-multi-virulence locus sequence (CRISPR-MVLST) with PFGE technique has been established and evaluated in *S.* Newport. The result demonstrated that both typing approaches have a high discrimination potential for tracking and clustering the source of outbreak of *S.* Newport (Shariat *et al.*, 2013). Because of the CRISPR-MVLST is a sequencing based technique, hence, this approach is still not practically for routine screening since it is unaffordable to most laboratories. All of this information suggested that the CRISPR arrays probably contribute the information helpful for serotyping of *S. enterica.*

Although several studies have been reported that the *S. enterica* contains 2 types of CRISPR arrays in the chromosomes, and application of CRISPR typing and subtyping for improved laboratory surveillance have been reported (Aarestrup *et al.*, 2003; Fabre *et al.*, 2012; Shariat *et al.*, 2013; Shariat *et al.*, 2015). There have been no report on the investigation of the CRISPR array system in *S. enterica* isolated from various sources in Thailand. Accordingly, the aim of this study was to investigate the diversity of the CRISPR arrays in *S. enterica* isolated from various sources and applying the *in silico* PCR-RFLP technique as a tracking tool for determining the relationship of *S. enterica* serotypes isolated from various sources.

MATERIALS AND METHODS

Bacterial samples

A total of 117 *S. enterica* samples in this study were derived from our previous study and were isolated from animal dung and meat samples (Lertworapreecha *et al.*, 2016). The feces samples were collected from 40 small-scale local farms as 100 from individual swine, 75 from pooled chicken, and 25 from individual cattle. Forty samples of both pork and chicken meat were collected from fresh markets in Phatthalung Province, Thailand, between June-December, 2014. Isolation, identification and serovar typing of *S. enterica* was carried out as described previously (Lertworapreecha *et al.*, 2016).

Amplification of CRISPR arrays

DNA extraction was carried out by picking approximately 2-3 colonies of *S. enterica* into a 1.5 mL microcentrifuge tube containing 200 μL of TE buffer (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0) and boiling for 10 minutes. DNA was stored in -20 °C until further use. CRISPR array amplification was performed using primers specific for CRISPR-I (F_CR-I: 5'-GCTGGTGAAACGTGTTTATCC-3'; R_CR-I: 5'-ATTCCGGTAGATYTKGATGGAC-3',) and CRIRPR-II (F_CR-II: 5'-AACGCCATGGCCTTCTCCTG-3'; R_CR-II: 5'-CAAAATCAGYAAATTAGCTGTTC-3') (Grissa *et al.*, 2007). Polymerization of target DNA was carried out using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, MA, USA). The reaction mixture (50 mL) composes of 1X PCR reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each specific CRISPR primer, and 2 Unit of DNA polymerase. Amplification condition was performed as described, initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 50 min, annealing at 50 °C for 1 min, primer extension at 72 °C for 1 min, and one cycle with a final extension step at 72 °C for 5 min. The amplification product was analyzed and visualized by 1.5 % agarose gel electrophoresis stained with a GelRed^{Tm} (Thermo Fisher Scientific, MA, USA).

CRISPR analysis

All PCR products from positive samples were purified and analyzed by nucleotide sequencing (Biobasic: Canada), and all sequences were analyzed for CRISPR-I and CRISPR-II by CRISPRFinder

program (Grissa *et al.*, 2007). The diversity of CRISPR-I, CRISPR- II and direct repeat were aligned using Bioedit (Hall, 2004) and MEGA7 program (Kumar *et al.*, 2016). The consensus sequence of direct repeat was constructed by WebLoGo (Crooks *et al.*, 2004).

In silico PCR-RFLP

The *in silico* PCR- RFLP of the CRISPR arrays were simulated by NEBcutter V2.0 (Vincze *et al.*, 2003), using the *Bst*UI restriction enzyme which recognizes and cuts at specific sequence (CG/CG) along the CRISPR. Determination of the DNA size was also analyzed by the software (NEBcutter V2.0).

RESULTS AND DISCUSSION

Detection and Variation of CRISPR-I and CRISPR-II in *S. enterica*

A total 117 of *S. enterica* isolated from various sources were subjected for detection of CRISPR-I and CRISPR-II. Of these, 48 (41%) and 71 (60.68%) isolates were PCR- positive for CRISPR- I and CRISPR- II, respectively. The numbers of spacers of the CRISPR-I and CRISPR-II were between 2-16 spacers and 1-17 spacers, respectively. The nucleotide length of the spacer in both CRISPR-I and CRISPR-II were between 30-37 nucleotides. The nucleotide length of the direct repeat in CRISPR-I and CRISPR-II were between 26-29 and 25-30 nucleotides, respectively (Table 1).

Table 1 *S. enterica* isolated from various sources, and number of spacer and direct repeat of the CRISPR-I and CRISPR-II.

Isolates No.	Source	Serovar	CRISPR-I	Number of Spacer	CRISPR length (bp)	DR length (bp)	CRISPR-II	Number of Spacer	CRISPR length (bp)	DR length (bp)
1	swine feces	Weltevreden	-				+	13	820	29
2	swine feces	Rissen	-				-			
3	swine feces	Weltevreden	+	8, 4	517, 276	29	+	15	943	29
4	swine feces	Weltevreden	-				+	8	516	29
5	swine feces	Weltevreden	-				+	15	944	29
6	swine feces	Rissen	-				-			
7	swine feces	Weltevreden	-				+	16	1005	29
8	swine feces	Stratford	-				+	15	943	26
9	swine feces	Typhimurium	-				+	15	941	25
10	swine feces	Sandown	+	14	881	29	-			
11	swine feces	Typhimurium	+	8, 7	517, 457	29	+	17	1058	27
12	swine feces	Typhimurium	+	14	883	29	+	14, 1	885, 86	29
13	swine feces	Cremieu	-				+	4	269	29
14	swine feces	Weltevreden	-				+	16	1004	29
15	swine feces	Typhimurium	+	8, 6	517, 397	29	+	15	947	29
16	swine feces	Paratyphi B	-				-			
17	swine feces	Weltevreden	-				+	15	943	29
18	swine feces	Weltevreden	-				+	16	1000	29
19	swine feces	Paratyphi B	+	7, 6	456, 398	29	+	16	1005	29
20	swine feces	Weltevreden	-				+	15	944	29
21	swine feces	Panama	-				+	9	577	29
22	swine feces	Weltevreden	+	15	940	29	+	15	943	29
23	swine feces	Weltevreden	-				+	17	1055	29
24	swine feces	Weltevreden	+	11	701	29	+	15	944	29
25	swine feces	Typhimurium	+	16	1004	26	+	15	945	29
26	swine feces	Typhimurium	-				-			
27	swine feces	Weltevreden	+	13	821	29	+	16	1002	29
28	swine feces	Braenderup	+	13	819	26	-			
29	swine feces	Rissen	-				-			
30	swine feces	Rissen	+	14	880	29	+	16	1003	29
31	swine feces	Saintpaul	+	7	456	29	-			
32	swine feces	Weltevreden	-				+	16	1004	29
33	swine feces	Amsterdam	-				+	16	1005	29
34	swine feces	Rissen	-				-			
35	swine feces	Amsterdam	-				+	13	822	30
36	swine feces	Typhimurium	-				-			
37	swine feces	Typhimurium	-				+	7, 6	456, 395	29
38	swine feces	Weltevreden	-				+	16	1007	29
39	chicken feces	Weltevreden	-				-			
40	chicken feces	Sandown	-				-			
41	chicken feces	Weltevreden	-				+	12	764	27
42	chicken feces	Fillmore	-				+	16	1003	29
43	chicken feces	Hadar	+	2	147	26	-			
44	chicken feces	Muenchen	-				-			
45	chicken feces	Hadar	-				-			
46	chicken feces	Choleraesuis	-				+	14	884	29
47	chicken feces	Hadar	+	15	947	29	+	16	1000	29
48	chicken feces	Braenderup	-				+	15	945	29

Table 1 (continued)

Isolates No.	Source	Serovar	CRISPR-I	Number of Spacer	CRISPR length (bp)	DR length (bp)	CRISPR-II	Number of Spacer	CRISPR length (bp)	DR length (bp)
49	chicken feces	Istanbul	+	16	1004	29	-			
50	chicken feces	Bardo	+	15	958	29	+	16	1006	29
51	chicken feces	Bardo	+	12	766	29	-			
52	chicken feces	Braenderup	-				+	16	1006	29
53	chicken feces	Braenderup	-				+	16	1004	29
54	chicken feces	Weltevreden	-				+	15	947	29
55	chicken feces	Weltevreden	+	6	396	29	+	16	1008	29
56	chicken feces	Magherafelt	-				-			
57	chicken feces	Braenderup	+	16	1005	29	+	16	1006	29
58	chicken feces	Braenderup	-				+	16	1006	29
59	chicken feces	Virginia	+	16	1004	29	-			
60	chicken feces	Braenderup	-				+	16	1005	29
61	chicken feces	Rissen	+	16	1026	29	+	16	1004	29
62	chicken feces	Weltevreden	+	9	569	30	+	15	942	29
63	chicken feces	Bardo	+	9	579	29	+	16	1001	25
64	chicken feces	Paratyphi B	+	2	147	26	-			
65	chicken feces	Glostrup	-				+	15	947	29
66	chicken feces	Mbandaka	-				+	15	944	29
67	chicken feces	Weltevreden	+	15	946	29	+	10	640	27
68	chicken feces	Hadar	+	16	1008	29	-			
69	cattle feces	Bardo	+	4	272	29	+	17	1063	29
70	cattle feces	Bardo	+	4	269	26	+	16	1004	29
71	cattle feces	Weltevreden	-				+	15	946	29
72	chicken meat	Typhimurium	-				+	15	945	29
73	chicken meat	Albany	-				-			
74	chicken meat	Albany	-				-			
75	chicken meat	Typhimurium	+	15	948	29	+	10	640	29
76	chicken meat	Kotu	-				-			
77	chicken meat	Albany	-				-			
78	chicken meat	Albany	-				-			
79	chicken meat	Weltevreden	+	14	885	29	+	11	696	27
80	chicken meat	Albany	-				-			
81	chicken meat	Typhimurium	-				-			
82	chicken meat	Typhimurium	+	16	1005	29	+	15	964	29
83	chicken meat	Albany	-				-			
84	chicken meat	Typhimurium	+	14	883	29	+	15	944	29
85	chicken meat	Albany	-				-			
86	chicken meat	Albany	-				+	16	1005	29
87	chicken meat	Albany	-				-			
88	chicken meat	Give	+	5	333	29	+	4	272	29
89	chicken meat	Albany	-				-			
90	chicken meat	Albany	-				-			
91	chicken meat	Typhimurium	+	14	883	29	+	13	822	29
92	chicken meat	Typhimurium	+	16	1009	29	+	15	944	29
93	vegetable	Typhimurium	+	14	883	29	-			
94	vegetable	Anatum	+	7	455	29	-			
95	vegetable	Anatum	+	7	455	29	+	12	761	29
96	vegetable	Weltevreden	+				-			
97	vegetable	Typhimurium	+				-			
98	vegetable	Weltevreden	-				-			
99	vegetable	Weltevreden	-				-			
100	vegetable	Weltevreden	-				-			
101	pork	Anatum	+	7	455	29	+	12	760	29
102	pork	Weltevreden	+	8	516	29	-			
103	pork	Rissen	-				-			
104	pork	Typhimurium	+	14	883	29	+	15	944	29
105	pork	Give	+	6	394	29	+	4	272	29
106	pork	Give	+	5	333	29	+	4	272	29
107	pork	Anatum	+	8	516	29	+	2	150	29
108	pork	Amsterdam	+	4	272	29	+	16	1005	29
109	pork	Weltevreden	+	15	942	29	-			
110	pork	Typhimurium	+	15	946	29	-			
111	pork	Weltevreden	+				-			
112	pork	Weltevreden	-				+	11	700	29
113	pork	Weltevreden	-				+	16	1006	29
114	pork	Weltevreden	-				+	16	1006	29
115	pork	Typhimurium	-				+	16	1005	29
116	pork	Weltevreden	-				-			
117	pork	Weltevreden	-				+	12	760	29
Total CRISPR positive			48(41%)				71 (60.68%)			

The direct repeat sequence alignment of CRISPR-I and CRISPR-II indicated that high diversity of single nucleotide polymorphisms (SNPs) found scattered in both CRISPR-I and CRISPR-II, which can be divided in 30 and 105 diverse patterns. Clustering the polymorphisms of the direct repeat of CRISPR-I by phylogenetic tree construction indicated that of all 30 patterns can be classified into 4 (A-D) groups (Figure 1). Whereas, among the 105 diverse patterns of CRISPR-II can be classified into 6 (A-F) groups (Figure 2).

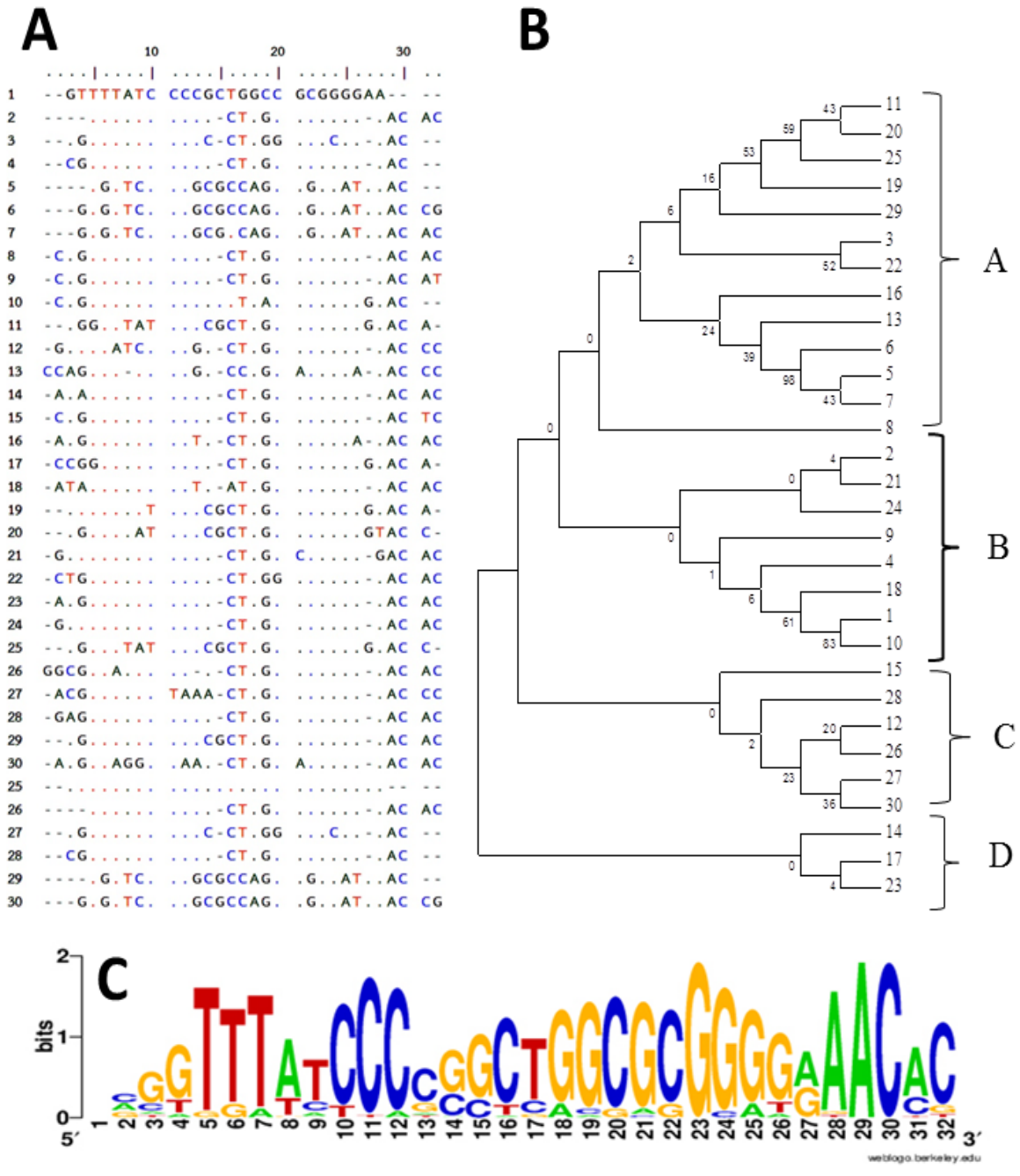

Figure 1 Diversity of the direct repeat of CRISPR-I isolated from *S. enterica*. A) Variation of nucleotide composition in the direct repeat from different sources of *S. enterica.* B) Nucleotide alignment and phylogenetic tree analysis of direct repeat (Neighbor joining with 1000 bootstrap: MEGA 6). C) Consensus sequence of direct repeat from different sources of *S. enterica* (constructed by WebLoGo).

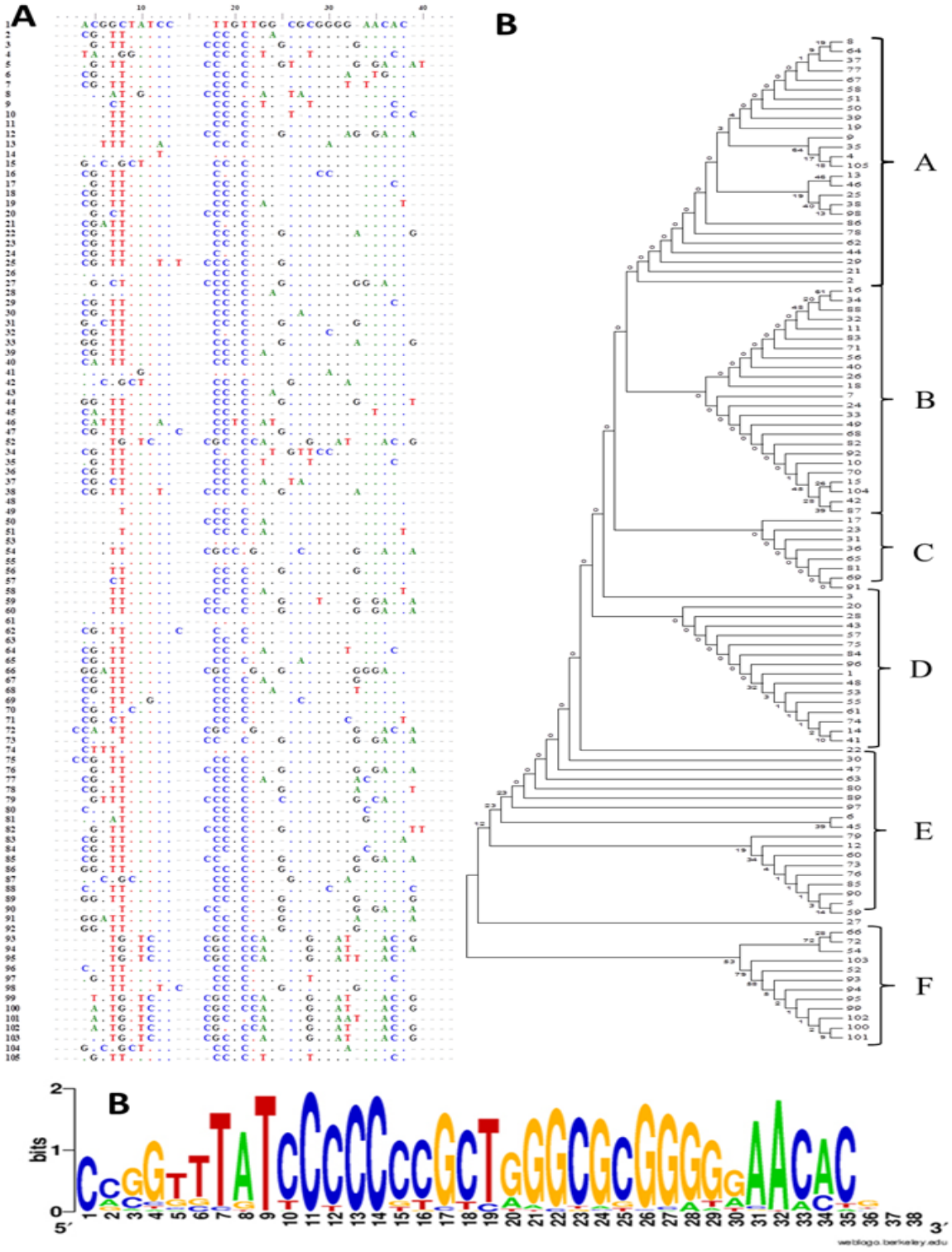

Figure 2 Diversity of the direct repeat of CRISPR-II isolated from *S. enterica*. A) Variation of nucleotide composition in direct repeat from different sources of *S. enterica.* B) Nucleotide alignment and phylogenetic tree analysis of direct repeat (Neighbor joining with 1000 bootstrap: MEGA 6). C) Consensus sequence of direct repeat from different sources of *S. enterica* (constructed by WebLoGo).

Alignment comparison of the CRISPR-I and CRISPR-II spacers sequences were further performed in S. Weltevreden as it is the most common serovar found in this study. The phylogenetic tree constructed from the sequence alignment of the CRISPR-I spacer clearly showed that S. Weltevreden was divided into two groups, corresponding to the source pig and chicken. In contrast to CRISPR-I, the phylogenetic tree constructed from sequence alignment of the CRISPR-II spacer was unable to discriminate between S. Weltevreden isolated from pig and chicken (Figure 3). There is an evidence indicated that the diversity of CRISPR array is the result of the accumulation of bacteriophage and plasmid invasion (Barrangou, 2015; Marraffini *et al.*, 2010). Furthermore, the number of spacers probably implies a variety of bacteriophage found in the environment (Sheludchenko *et al.*, 2015). The number of spacers in CRISPR-I and CRISPR-II were different; our study found that CRISPR-II has diverse spacers in terms of number and nucleotide variation more than those in CRISPR-I. This finding probably due to the fact that CRISPR-II is composed of Cas9 protein, which cleave the target DNA more efficiently than other Cas proteins found in CRISPR-I (Deltcheva *et al.*, 2011; Jinek *et al.*, 2012).

In silico PCR-RFLP

The nucleotide sequence analysis of the CRISPR-I of S. Weltevreden revealed a high diversity in the number and nucleotides of the direct repeat region as well as the nucleotide sequence of the spacer region. It is possible to apply the nucleotide sequence of CRISPR-I as an epidemiological marker. The results of *in silico* PCR-RFLP using the *Bst*UI restriction enzyme and simulation separating in high resolution gel electrophoresis (Spreadex® gels) demonstrated that the enzyme was able to generate several DNA banding patterns. Interestingly, these different patterns correspond to S. Weltevreden isolated from different sources. Most of isolates from swine showed closely related DNA banding patterns indicated that it probably originated from the same lineage (Figure 4).

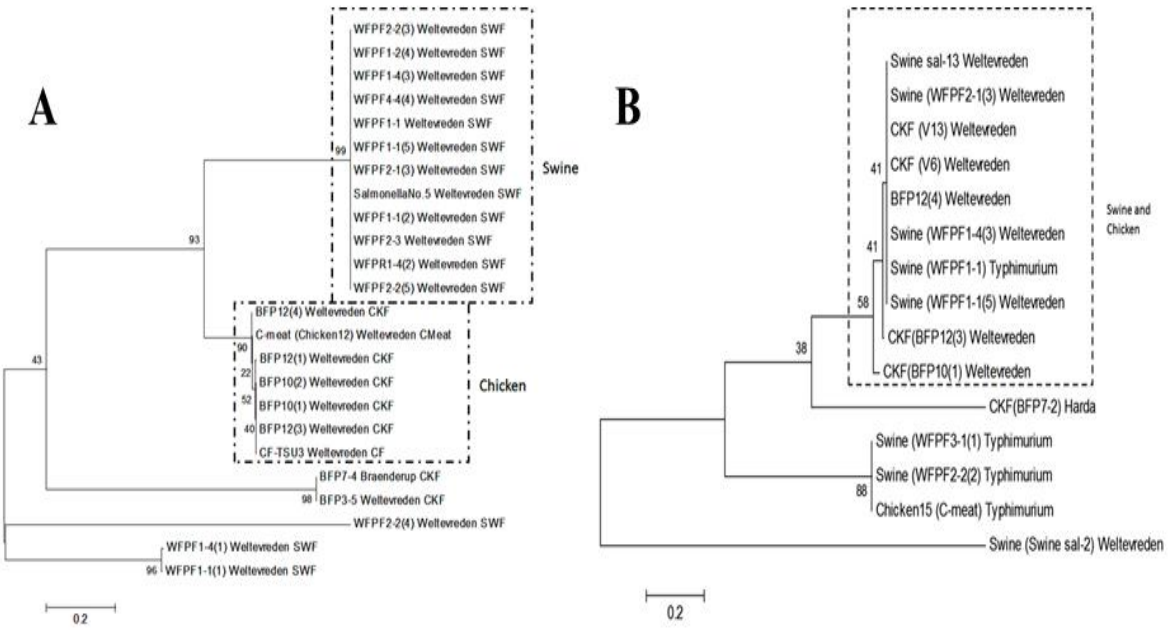

Figure 3 Comparison of CRISPR-I (A) and CRISPR-II (B) spacers in S. Weltevreden. The phylogenetic tree was constructed by MEGA 6 (Neighbor joining with 1000 bootstrap). Numbers next to each node represents percent support for the node. Scale bar represents fractional amount of genetic change.

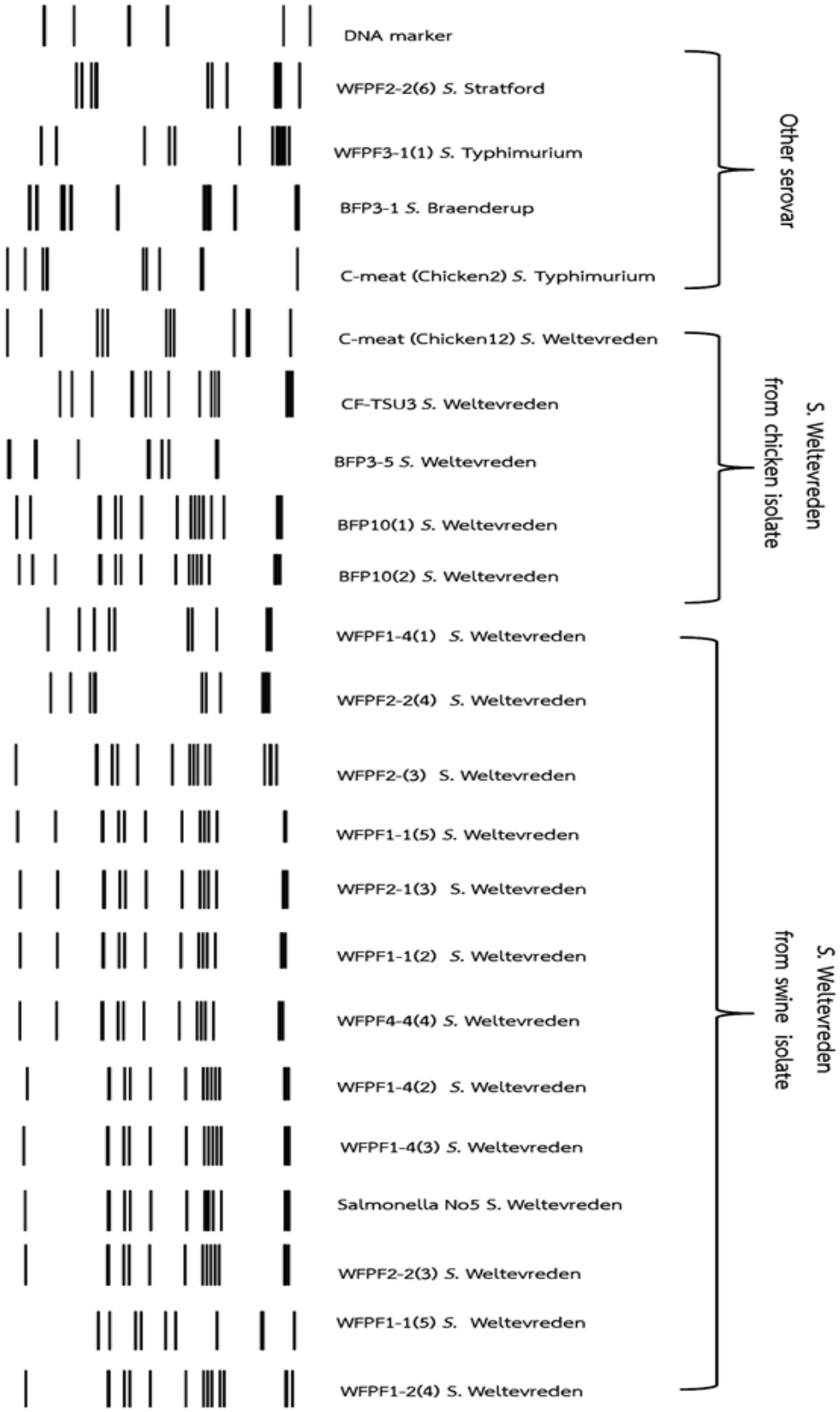

Figure 4 *In silico* PCR-RFLP of CRISPR-I of S. Weltevreden isolated from different sources, digested by *Bst*UI and simulation separating in high resolution gel electrophoresis (Spreadex® gels).

A diversity of CRISPR systems were also found in Gram-positive bacteria, such as *Streptococcus thermophilus*, lactic acid bacteria and *Bifidobacterium*, which can be used as a genotyping tool in those bacterial species (Briner *et al.*, 2015; Horvath *et al.*, 2008; Sheludchenko *et al.*, 2015). A large and differing CRISPR cluster exhibit in these systems are likely dynamic and suggesting that they are important for survival and evolutionary relatedness in those bacteria. The CRISPR polymorphisms have also been used for the serological distinction of *Shigella* subtypes. However, the results indicated that the method may not be specific enough to distinguish each subtype because of the large variety of *Shigella* serotypes. Interestingly, the results suggested that CRISPR analysis able to use as a tool for identification of *Shigella* species (Yang *et al*., 2015).

Taken together, our finding was different from the previous study in *Shigella*. Based on the findings, CRISPR arrays of S. *enterica* are diverse, allowing them to be used as an alternative tool for tracking the *S. enterica* outbreak. Although this is study reports an *in silico* simulation method, it indicates that the nucleotide sequences, and, thus, the results can be reliable and feasible.

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REFERENCES

Aarestrup FM, Lertworapreecha M, Evans MC, Bangtrakulnonth A, Chalermchaikit T, Hendriksen RS, & Wegener HC (2003) Antimicrobial susceptibility and occurrence of resistance genes among *Salmonella enterica* serovar Weltevreden from different countries. J Antimicrob Chemother 52: 715–718.

Barrangou R (2015) Diversity of CRISPR-Cas immune systems and molecular machines. Genome Biol 16: 247. doi: 10.1186/s13059-015-0816-9

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, & Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709–1712.

Briner AE, Lugli GA, Milani C, Duranti S, Turroni F, Gueimonde M, Margolles A, van Sinderen D, Ventura M, & Barrangou R (2015) Occurrence and diversity of CRISPR-Cas systems in the genus *Bifidobacterium*. PLoS One 10. doi: ARTN e013366110.1371/journal.pone.0133661

Crooks GE, Hon G, Chandonia JM, & Brenner SE (2004) WebLogo: A sequence logo generator. Genome Res 14: 1188–1190.

Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao YJ, Pirzada ZA, Eckert MR, Vogel J, & Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471: 602–607.

Fabre L, Zhang J, Guigon G, Le Hello S, Guibert V, Accou-Demartin M, de Romans S, Lim C, Roux C, Passet V, Diancourt L, Guibourdenche M, Issenhuth-Jeanjean S, Achtman M, Brisse S, Sola C, & Weill FX (2012) CRISPR typing and subtyping for improved laboratory surveillance of *Salmonella* infections. PLoS One 7: e36995. doi: 10.1371/journal.pone.0036995

Grissa I, Vergnaud G, & Pourcel C (2007b) CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res 35: W52–57.

Groenen PM, Bunschoten AE, van Soolingen D, & van Embden JD (1993) Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Mol Microbiol 10: 1057–1065.

Hall T. (2004). BioEdit version 7.0.0 http://www.mbio.ncsu.edu/BioEdit/BioDoc.pdf (March 22, 2017).

Horvath P, & Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327: 167–170.

Horvath P, Romero DA, Coute-Monvoisin AC, Richards M, Deveau H, Moineau S, Boyaval P, Fremaux C, & Barrangou R (2008) Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. J Bacteriol 190: 1401–1412.

Iranzo J, Lobkovsky AE, Wolf YI, & Koonin EV (2013) Evolutionary dynamics of the prokaryotic adaptive immunity system CRISPR-Cas in an explicit ecological context. J Bacteriol 195: 3834–3844.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, & Charpentier E (2012) A Programmable dual-

RNA- guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816–821.

Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, & van Embden J (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35: 907–914.

Kauffmann F (1971) On the classification and nomenclature of the genus *Salmonella*. Acta Pathol Microbiol Scand B Microbiol Immunol 79: 421–422.

Koonin EV, & Makarova KS (2013) CRISPR- Cas: evolution of an RNA- based adaptive immunity system in prokaryotes. RNA Biol 10: 679–686.

Kumar S, Stecher G, & Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7. 0 for bigger datasets. Mol Biol Evol 33: 1870–1874.

Lertworapreecha. M, Noomee. S, Sutthimusik. S, Utarapichat. B, & Tontikapong. K (2016) Multidrug resistant and extended spectrum b-lactamase producing *Salmonella enterica* isolated from food animals in Phatthalung, Thailand. Southeast Asian J Trop Med and Public Health 47: 1257–1269.

Marraffini LA, & Sontheimer EJ (2010) CRISPR interference: RNA- directed adaptive immunity in bacteria and archaea. Nat Rev Genet 11: 181–190.

Pougacha KS, Lopatina AV & Severinov KV (2012) CRISPR adaptive immunity systems of procaryotes. Mol Biol (Mosk) 46: 195–203.

Pourcel C, Andre-Mazeaud F, Neubauer H, Ramisse F & Vergnaud G (2004) Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. BMC Microbiol 4: 22. doi: 10.1186/1471-2180-4–22.

Pourcel C, Salvignol G, & Vergnaud G (2005) CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151: 653–663.

Rath D, Amlinger L, Rath A, & Lundgren M (2015) The CRISPR- Cas immune system: Biology, mechanisms and applications. Biochimie 117: 119–128.

Schouls LM, Reulen S, Duim B, Wagenaar JA, Willems RJL, Dingle KE, Colles FM, & Van Embden JDA (2003) Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: Strain diversity, host range, and recombination. J Clin Microbiol 41: 15–26.

Shariat N, Kirchner MK, Sandt CH, Trees E, Barrangou R & Dudley EG (2013) Subtyping of *Salmonella enterica* serovar Newport outbreak isolates by CRISPR- MVLST and determination of the relationship between CRISPR- MVLST and PFGE results. J Clin Microbiol 51: 2328–2336.

Shariat N, Timme RE, Pettengill JB, Barrangou R & Dudley EG (2015) Characterization and evolution of *Salmonella* CRISPR-Cas systems. Microbiology 161: 374–386.

Sheludchenko MS, Huygens F, Stratton H & Hargreaves M (2015) CRISPR diversity in *E. coli* isolates from Australian animals, humans and environmental waters. PLoS One 10: e0124090, doi: 10.1371/journal.pone.0124090.

Terns MP & Terns RM (2011) CRISPR-based adaptive immune systems. Curr Opin Microbiol 14: 321–327.

van der Oost J, Jore MM, Westra ER, Lundgren M & Brouns SJ (2009) CRISPR- based adaptive and heritable immunity in prokaryotes. Trends Biochem Sci 34: 401–407.

Vincze T, Posfai J & Roberts RJ (2003) NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res 31: 3688–3691.

Yang C, Li P, Su W, et al. (2015) Polymorphism of CRISPR shows separated natural groupings of *Shigella* subtypes and evidence of horizontal transfer of CRISPR. RNA Biol 12: 1109–1120.

Zou W, Lin WJ, Foley SL, Chen CH, Nayak R & Chen JJ (2010) Evaluation of pulsed- field gel electrophoresis profiles for identification of *Salmonella* serotypes. J Clin Microbiol 48: 3122–3126.

Zou W, Lin WJ, Hise KB, Chen H C, Keys C, & Chen JJ (2012) Prediction system for rapid identification of *Salmonella* serotypes based on pulsed- field gel electrophoresis fingerprints. J Clin Microbiol 50: 1524–1532.