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Original Article

Modified Method of Multilocus Sequence Typing (MLST) for Serotyping in *Salmonella* Species

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Multilocus sequence typing (MLST) of *Salmonella* is useful method for replacing serotyping using antisera but is limited by difficulties associated with in polymerase chain reaction (PCR). We optimized the PCR reaction, especially annealing temperature and extension time (94℃ for 2 min; 40 cycles at 94℃ for 30 sec, 56.8℃ for 1 min, 72℃ for 2 min; and 72℃ for 10 min). The degradation of PCR product by thermostable nucleases was inhibited by using template DNAs treated proteinase K or purified by a commercialized preparation kit. The resulting modified MLST was used as accurate and fast typing method.

Key Words: Multilocus sequence typing (MLST), *Salmonella* spp., PCR conditions, Serotyping

INTRODUCTION

The genus *Salmonella* is important as a global water- and food-borne pathogen. *Salmonella* consists of two species, six subspecies, and 2,579 serovars according to the Kauffman-White scheme (1). Each serotype was defined by the combination of the O-antigen and the two H-antigens, which were tested using antisera. It is sometimes difficult to determine the serotype when variation in the somatic antigen (rough, mucoid) and flagellar antigens occur (2). In this case, MLST is a very useful method for replacing serotyping using antisera. MLST is determined with sequence types (STs) that are obtained by alleles number of 7 housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *thrA*, *sucA*, and *purE*) in DNA sequence database (http://mlst.warwick.ac.uk/mlst). However, PCR conditions sometimes induce inappropriate PCR products which can appear as poor quality, unclean, and smeared bands on an agarose gel (3). In addition, the amplified PCR products are sometimes degraded by thermostable nucleases (4). For this reason, we modified the MLST method by optimizing the PCR conditions and inhibiting the degradation of the PCR product by the endogenous *Salmonella* nuclease.

MATERIALS AND METHODS

Bacterial strains and DNA preparation

The bacterial strains used in this study were *S.* Typhimurium ATCC 14028, which was used to optimize the PCR conditions, and twenty serovars of *Salmonella*, which were used to confirm the success of the modified MLST method. A boiling method was used to prepare the

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Table 1. Oligonucleotide sequences of primers for multi-locus sequence type

Primers		Oligonucleotide sequences (5' → 3')	Expected Tm. (℃)	Product size (bp)
aroC	F	CCTGGCACCTCGCGCTATAC	64.6	826
	R	CCACACACGGATCGTGGCG	63.8	
dnaN	F	ATGAAATTTACCGTTGAACGTGA	57.5	833
	R	AATTTCTCATTCGAGAGGATTGC	59.3	
hemD	F	ATGAGTATTCTGATCACCCG	56.4	666
	R	ATCAGCGACCTTAATATCTTGCCA	61.8	
hisD	F	GAAACGTTCCATTCCGCGCAGAC	66.4	894
	R	CTGAACGGTCATCCGTTTCTG	61.3	
thrA	F	GTCACGGTGATCGATCCGGT	62.5	852
	R	CACGATATTGATATTAGCCCG	57.4	
sucA	F	AGCACCGAAGAGAAACGCTG	60.5	643
	R	GGTTGTTGATAACGATACGTAC	58.4	
purE	F	ATGTCTTCCCGCAATAATCC	56.4	510
	R	TCATAGCGTCCCCCGCGGATC	67.2	

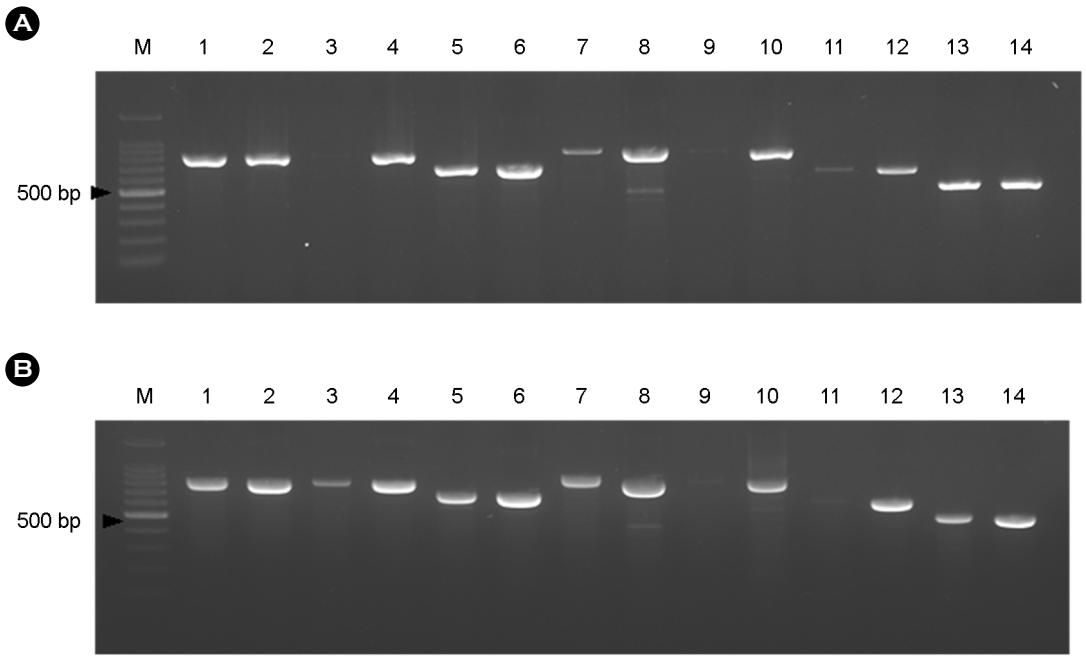

Figure 1. Comparison of the amplified PCR products using two commercialized PCR premix kits. Aliquots of 5 μl of PCR products were analyzed on a 1.5% agarose gel at 100 volts for 30 min. The PCR products with the optimized condition showed thicker and cleaner bands than those obtained by the previous condition with two different kits. (A) iNtRON Co., Ltd. PCR premix, Korea (B) SNC Co., Ltd PCR premix, Korea. M: 100 bp of DNA marker; Lanes 1~2, *aroC*, amplified with the previous and optimized conditions; Lanes 3~4: *dnaN*, amplified with the previous and optimized conditions; Lanes 5~6, *hemD*, amplified with the previous and optimized conditions; Lanes 7~8, *hisD*, amplified with the previous and optimized conditions; Lanes 9~10, *thrA*, amplified with the previous and optimized conditions; Lanes 11~12, *sucA*, amplified with the previous and optimized conditions; Lanes 13~14, *purE*, amplified with the previous and optimized conditions.

template DNA for the PCR reaction. In brief, bacterial strains were cultured on tryptic soy agar (Difco, Detroit, MI, USA), and three colonies were picked and suspended in 50 μl of distilled water. The suspension was boiled at 100℃ for 10 min and centrifuged at 16,000 × g for 5 min. The supernatant was collected and stored at -20℃ until ready for use.

The inhibition of PCR product degradation was evaluated using three different template DNAs; The boiling supernatant with and without proteinase K treatment, and chromosomal DNA purified using a commercialized preparation kit (QIAamp UCP Pathogen Mini kit, Qiagen, Germany). Proteinase K was used at different concentrations (0.1, 0.2, 0.4, 0.8, and 1.2 mg/μl) at 37℃ for 1 h and then inactivated by heating the reaction mixture to 75℃ for 20 min.

PCR amplification

The primers in this study were recommended pairs of 7 housekeeping genes on the MLST website (http://mlst.warwick.ac.uk/mlst) (Table 1), and the PCR components were used two commercialized PCR premixes (Maxime PCR premix, iNtRON Co., Ltd., Korea; iMOD PCR premix, SNC Co., Ltd., Korea). To optimize the PCR conditions, we used different concentrations of the template DNA (0.25, 0.5, 1, 2.5, 5, 10, and 25 ng/μl), annealing temperatures (50~60℃), and extension times (0.5~3 min). The amplified PCR products were stored at 4℃ for 3 days and analyzed on a 1.5% agarose gel at 100 volts for 30 min with the stain of fluorescent dye, SafeView™ (abm Inc., Richmond City,

Table 2. Alleles numbers and sequence types of 20 strains of *Salmonella* serovars

No.	Serovar	*aroC*	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	Sequence types
1	Enteritidis	5	2	3	7	6	6	11	11
2	Typhimurium	10	7	12	9	5	9	2	19
3	I 4,[5]12:i:-	10	19	12	9	5	9	2	34
4	Montevideo	43	41	16	42	35	13	4	81
5	Virchow	2	7	10	10	8	10	14	38
6	Infantis	17	18	22	17	5	21	19	32
7	Typhi	1	1	2	1	1	1	5	2
8	Bareilly	81	5	101	12	124	130	17	362
9	Thompson	14	13	18	12	14	18	1	26
10	Newport	2	2	15	14	15	20	12	31
11	Saintpaul	5	14	18	9	6	12	17	27
12	Stanley	16	16	26	18	8	12	18	51
13	ParatyphiA	45	4	8	44	27	9	8	85
14	Agona	3	3	7	4	3	3	7	13
15	ParatyphiB	2	14	24	14	37	19	8	86
16	Braenderup	12	2	15	14	11	14	16	22
17	Rissen	92	107	79	156	64	151	87	469
18	Mbandaka	15	70	93	78	113	6	68	413
19	Panama	22	11	25	21	10	23	23	48
20	Anatum	10	14	15	31	25	20	33	64

BC, Canada).

Sequencing and MLST

Salmonella serovar results were simultaneously compared with those obtained by serotyping using antisera and with the modified MLST method using the optimized PCR conditions. Sequencing of the nucleotides was performed with an automated sequencer (ABIPRISM 3730XL, Foster City, CA, USA), and the sequence type was determined using analysis tools on the MLST website. We applied the modified MLST method to 20 serovar of *Salmonella* isolates, which were the most prevalent serotypes in outbreaks in Korea during the previous 3 years (2011~2013) (Table 2).

RESULTS

Stability of PCR product

The stability of the PCR products was shown to be different according to their preparation method. When stored at 4℃ during 24 h, the PCR product using boiling supernatant untreated proteinase K was completely degraded. However, the products obtained using the other DNA was maintained without degradation until 72 h (data not shown). A total of 0.1~0.8 mg/μl of proteinase K was sufficient to treat the template DNA using the boiling method. The PCR reaction didn't perform properly in high concentrations of proteinase K more than 1.2 mg/μl (data not shown).

Modification and confirmation of MLST method

The PCR condition was optimized at 94℃ for 2 min; 40 cycles at 94℃ for 30 sec, 56.8℃ for 1 min, 72℃ for 2 min; and 72℃ for 10 min. It was determined that 2.5~5 ng/μl of template DNA was the optimal concentration for all samples except for *thrA* (0.25~1 ng/μl). The PCR amplification was disturbed by high concentrations of template DNA more than 25 ng/μl (data not shown). The optimized PCR condition was compared with the previous condition using different two commercialized PCR premix kit. Using both kits, PCR products obtained under the optimized PCR condition showed thicker and cleaner bands than those obtained by the previous method on 1.5% agarose gel (Fig. 1).

When comparing the modified MLST with the serotyping using antisera, the sequence type obtained by MLST was consistent with the result obtained by serotyping based on the Kauffman-White scheme (Table 2).

DISCUSSION

Serotyping using antisera is the gold standard by which to determine the *Salmonella* serovar but it is time consuming, requires skill, and is an expensive method requiring too many antisera. In contrast, MLST is relatively easy, fast, and cheap and is only performed using a PCR reaction considering the nucleotide sequence. Several reports have previously presented different PCR conditions, but they did not completely compensate for the previous method (5~10). We optimized the PCR condition and treated the samples with proteinase K during the template DNA preparation to improve the MLST efficacy. The PCR condition was optimized at 94℃ for 2 min; 40 cycles at 94℃ for 30 sec, 56.8℃ for 1 min, 72℃ for 2 min; and 72℃ for 10 min. The degradation of PCR product by thermostable nucleases was inhibited by using template DNAs treated proteinase K or purified by a commercialized preparation kit. The sequence type obtained by modified MLST method in 20 serovars of *Salmonella* was consistent with the result obtained by serotyping based on the Kauffman-White scheme. The modified MLST will be used as accurate and fast typing method without trial error.

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