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Development of Multiple-Locus Variable-Number Tandem-Repeat Analysis for Molecular Subtyping of *Campylobacter jejuni* by Using Capillary Electrophoresis

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***Campylobacter jejuni* is a common cause of the frequently reported food-borne diseases in developed and developing nations. This study describes the development of multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) using capillary electrophoresis as a novel typing method for microbial source tracking and epidemiological investigation of *C. jejuni*. Among 36 tandem repeat loci detected by the Tandem Repeat Finder program, 7 VNTR loci were selected and used for characterizing 60 isolates recovered from chicken meat samples from retail shops, samples from chicken meat processing factory, and stool samples. The discrimination ability of MLVA was compared with that of multilocus sequence typing (MLST). MLVA (diversity index of 0.97 with 31 MLVA types) provided slightly higher discrimination than MLST (diversity index of 0.95 with 25 MLST types). The overall concordance between MLVA and MLST was estimated at 63% by adjusted Rand coefficient. MLVA predicted MLST type better than MLST predicted MLVA type, as reflected by Wallace coefficient (Wallace coefficient for MLVA to MLST versus MLST to MLVA, 86% versus 51%). MLVA is a useful tool and can be used for effective monitoring of *C. jejuni* and investigation of epidemics caused by *C. jejuni*.**

Campylobacter infection is one of the most commonly identified bacterial causes of acute gastroenteritis in humans worldwide (1). *C. jejuni* is the predominant species in the genus *Campylobacter* and is associated with human food-borne diseases. Usual symptoms of the infection caused by *C. jejuni* are fever, diarrhea, and abdominal cramps. Although infection with *Campylobacter* usually is not fatal, the reported cases of campylobacteriosis often exceed those of infections caused by the *Salmonella* species or *Escherichia coli* (2). In Japan, *Campylobacter* is one of the three main causes of food-borne diseases, with the estimated number of cases being around 1.5 million persons per year (3). Poultry products often are contaminated with *C. jejuni*, and most of the infections are found to be associated with the handling of raw poultry or eating raw or undercooked poultry meat (4, 5).

Strain subtyping by molecular methods provides a powerful tool for epidemiological investigation and tracking the source of contamination (30, 31). To date, typing of *C. jejuni* strains was performed by random amplified polymorphic DNA analysis (8), amplified fragment length polymorphism (9), pulsed-field gel electrophoresis (10), ribotyping (9), *flaA* short variable region typing (11), microarray comparative genomic hybridization (12), repetitive sequence-based PCR fingerprinting (13), multilocus sequence typing (MLST) (14, 15), and whole-genome sequencing (WGS) (16). One of the most commonly used methods for *C. jejuni* typing in current research is MLST, which is considered the gold standard for the subtyping of *C. jejuni*. MLST of *C. jejuni* utilizes the sequence data obtained from seven housekeeping genes. The alleles from these housekeeping genes are assigned allele numbers based on a complete match to an allele in the global database, and the combination of these allele numbers makes up a sequence type and clonal complex. MLST is highly reproducible, and the data produced by this method are unambiguous due to an internationally standardized nomenclature. The results can be used for the construction of international databases that can be electronically exchanged. However, the major drawbacks of MLST lie in the fact that it is expensive, labor-intensive, and time-consuming because of the requirement for sequencing 7 genes.

The importance of identifying and eliminating the sources of *C. jejuni* contamination in order to reduce the risk of human exposure has compelled the need for rapid and reliable subtyping methods for *C. jejuni*. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) is a proven and highly discriminatory subtyping method for many food-borne pathogens, such as *Salmonella* (17), *E. coli* O157:H7 (7), *Listeria monocytogenes* (18), *Enterobacter sakazakii* (19), *Staphylococcus aureus* (20), and *Vibrio parahaemolyticus* (21). The method is based on the variation in the number of tandem repeated sequences found in many different loci in the genome of bacteria. VNTRs are short segments of DNA that have variable copy numbers. It is thought that the variation in copy number is due to DNA polymerase slippage during replication (21). Despite mutations that may occur within the tandem repeat, the unit length remains relatively constant while the copy number varies. The difference in copy numbers at specific loci is used to measure relatedness of strains in this sub-

Received 7 April 2015 **Accepted** 21 May 2015

Accepted manuscript posted online 29 May 2015

Citation Techaruvichit P, Takahashi H, Vesaratchavest M, Keeratipibul S, Kuda T, Kimura B. 2015. Development of multiple-locus variable-number tandem-repeat analysis for molecular subtyping of *Campylobacter jejuni* by using capillary electrophoresis. Appl Environ Microbiol 81:5318–5325. doi:10.1128/AEM.01151-15.

Editor: C. A. Elkins

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doi:10.1128/AEM.01151-15

typing scheme. Therefore, specific loci that are unique to a particular bacterial strain and contain VNTR are selected as MLVA markers. In brief, the VNTR loci first are PCR amplified. PCR products subsequently are separated on an agarose gel, by capillary electrophoresis, or on an automated capillary DNA sequencer. The number of tandem repeats is assessed based on the size of the PCR products. The MLVA profile is defined by the number of tandem repeats of the VNTR loci. Each unique MLVA profile coded by a multidigit is assigned a MLVA type number.

To date, there are no reports that describe the application of MLVA for the subtyping of *C. jejuni*. The challenge in the subtyping of *C. jejuni* using MLVA is that its genome sequence has a limited copy number of the TR. Most of the TR loci found by the Tandem Repeat Finder program (version 11.0) (22) showed around two copies of the tandem sequence. These were not likely to be polymorphic and would not have provided sufficient discriminatory power for determining the MLVA profiles. This finding complicated the initial stages of MLVA profiling. However, when the MLVA profile of *C. jejuni* ultimately was developed, it worked well for the subtyping of *C. jejuni* in this study. MLVA requires significant time to develop a specific MLVA assay for each organism. However, it has several advantages over other typing methods. MLVA is easy to perform at low costs, offering rapid typing with high discriminatory power, and moderate expertise is required. MLVA also is appropriate to type a large number of isolates and to be used in the laboratory for microbiological analysis in food factories.

This study described the development of the MLVA subtyping scheme for *C. jejuni* and the application of MLVA for comparing the efficiency of MLVA and MLST techniques for the subtyping of *C. jejuni*.

MATERIALS AND METHODS

Bacterial isolates. A total of 60 *C. jejuni* isolates were used in this study. The isolates were collected from chicken meat samples from retail shops and chicken meat samples and chicken cecum samples, as well as environmental swabs, from a chicken meat processing factory. The chicken meat samples from retail shops were collected on different days from various retail shops in Japan, which were distant from each other and supplied by different suppliers. The isolates from a chicken meat factory were from the strain collection at the Department of Veterinary Public Health, Chulalongkorn University. They were from a factory in Thailand from 2011 to 2013. The sampling date and the processed batches were specifically selected to ensure a diverse pool of *C. jejuni* isolates. In addition, *C. jejuni* ATCC 33560 (from bovine feces) and JCM2013 (from diarrheic stool sample of a child) were included for strain diversity. Out of the 60 isolates, 10 different isolates of *C. jejuni* collected from 10 different sampling locations, including *C. jejuni* ATCC 33560 and *C. jejuni* JCM2013, as well as isolates from the chicken meat samples in retail shops, were used to screen for potential VNTR loci.

DNA extraction. *C. jejuni* isolates were recovered from −85°C storage and grown on *Campylobacter* charcoal differential agar (CCDA) (Oxoid, Basingstoke, Hampshire, England). The plates were incubated at 42°C for 48 h under microaerophilic atmosphere generated by AnaeroPack-MicroAero (Mitsubishi Gas Chemical, Tokyo, Japan). Genomic DNA of the bacteria was extracted using a NucleoSpin tissue kit (TaKaRa, Otsu, Japan) per the manufacturer's instructions. Total DNA isolated was quantified using a Malcom e-spect spectrophotometer (Malcom, Tokyo, Japan) and stored at −20°C.

Identification of the TRs. The Tandem Repeat Finder program (version 11.0) (22) was used to identify the TRs in the 12 completed genome sequences of *C. jejuni* submitted to the DDBJ database (accessed on 2 May 2014). More than one hundred TRs were identified, out of which 36 TR loci with more than two TR sequence units were selected, except for loci V11 and V12, which had two TR units (Table 1). To screen for variability in the number of TR, PCR primers binding to both sides of the repeats were designed manually. These primers were used to amplify DNA from a set of 10 *C. jejuni* isolates of 10 diverse origins. TR loci containing variable numbers of TR then were chosen for MLVA typing.

MLVA typing. The 7 TR loci shortlisted for MLVA were amplified in the DNA isolated from the 60 *C. jejuni* isolates by PCR. The PCR was performed in a total volume of 50 μl containing 25 ng of DNA, 10× PCR buffer, 1 U of *Taq* DNA polymerase, 0.2 mM deoxynucleoside triphosphates, and 1,000 nM (each) forward and reverse primer. The PCR conditions were the following: initial denaturation at 94°C for 4 min; cycling at 94°C for 30 s, specific annealing (specific temperature for each locus is mentioned in Table 2) for 30 s, and extension at 72°C for 1 min for 35 cycles; and a final extension at 72°C for 10 min. The amplification product (5 μl) was loaded onto a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized under UV light. To analyze the variants further, the observed amplicons were subjected to capillary electrophoresis (CE; QIAxcel Advanced; Qiagen, Tokyo, Japan) for fragment analysis. The assessed PCR product size was used to calculate the number of tandem repeats in each locus. The flanking regions with known sizes were subtracted from the PCR product size, which results in the net size of the repeat region. The number of tandem repeats then was obtained by dividing the size of the repeat region by the repeat unit size. Finally, the PCR products which presented the copy number variants of the TR were sequenced to ensure the accuracy of the number of tandem repeats.

DNA sequencing of PCR products. To confirm that the variations in the length of the amplicons were the result of copy number variation, all of the PCR products obtained from the set of 10 *C. jejuni* strains used to screen for the variants and the PCR products of 50 *C. jejuni* isolates representing the copy number variants of the TR (previously analyzed by CE) were sequenced using the same primers as those used to amplify the VNTRs. Sequencing reactions were performed using the BigDye Terminator technology according to the manufacturer's instructions (Life Technologies). The products were analyzed using a 3130 Genetic Analyzer (Life Technologies). Sequences obtained using the forward and reverse sequencing primers were aligned using the Genetyx software (version 11; Genetyx Corp., Tokyo, Japan).

MLST typing. Based on the work of Dingle et al. (14), seven housekeeping genes (*aspA*, *glnA*, *glt*, *glyA*, *pgm*, *tkt*, and *uncA*) obtained from the set of 50 *C. jejuni* isolates were amplified and sequenced. The alleles and the sequence types are defined on the MLST website (http://pubmlst.org/campylobacter/).

Data analysis. Simpson's index of diversity and the degrees of congruence between MLVA and MLST subtyping schemes were determined via an online tool (http://www.comparingpartitions.info/). A diversity index (DI) of 1.0 indicates that a typing method was able to distinguish each isolate of a strain from all of the other isolates in the collection. The congruence coefficients were calculated using the adjusted Rand and Wallace coefficients; the adjusted Rand coefficient shows the quantitative evaluation of the overall congruence between two subtyping methods (23), whereas the Wallace coefficient is a directional congruence indicating the probability that isolates clustered together by one method also will cluster together when typed by the other method (24).

Nucleotide sequence accession numbers. The DDBJ accession numbers of *C. jejuni* strain NCTC11168, ICDCCJ07001, 81116, 00-2544, 81-176, IA3902, 00-2425, RM1221, PT14, doylei 269.97, S3, and M1 are AL111168, CP002029, CP000814, CP006709, CP000538, CP001876, CP006729, CP000025, CP003871, CP000768, CP001960, and CP001900, respectively.

RESULTS AND DISCUSSION

Identification of VNTR loci in *C. jejuni*. Since shorter repeats show a higher copy number and are more likely to be polymorphic

TABLE 1 Thirty-six initially selected VNTR loci found with Tandem Repeat Finder and tandem repeat information

Locus	Tandem repeat sequence	Repeat unit length (bp)	No. of TR in reference strain	No. of variants[a]	Coding region (according to NCBI database)	Position	Reference strain	Comment[d]
V1	TCTATCTTTGTATTATTAAGA	21	9.4		Hypothetical protein	1370374–1370570	NCTC11168	No amplified product in most test strains
V2[b]	AAAGAAAAAAAT	12	5.9	5	Noncoding	44616–44690	NCTC11168	Variable in tandem repeat
V3	TTTTAATAATATA	13	3.7	0	Noncoding	1091732–1091783	NCTC11168	Invariable in tandem repeat
V4	AAAGTAAAG	9	3.3	2	Hypothetical protein	765323–765352	NCTC11168	Invariable in tandem repeat[e]
V5	CGATGCAAA	9	3	0	Lipoprotein thioredoxin	1588706–1588732	NCTC11168	Invariable in tandem repeat
V6[b]	ATTAAT	6	3	2	ATP/GTP-binding protein	1409281–1409298	NCTC11168	Variable in tandem repeat
V7	TGAAAAAGAACTAAA	15	2.8	0	Noncoding	1326362–1326403	NCTC11168	Invariable in tandem repeat
V8	TTTTTATAGTTTTTACTT	18	2.4	0	Type I phosphodiesterase/ nucleotide pyrophosphatase	684675–684718	NCTC11168	Invariable in tandem repeat
V9	GCTTTGCTTTTG	12	2.3	0	Prolipoprotein diacylglyceryl transferase	371800–371826	NCTC11168	Invariable in tandem repeat
V10	TTAAATTCAAGC	12	2.1	0	Fibronectin/fibrinogen-binding protein	1281533–1281557	NCTC11168	Invariable in tandem repeat
V11[b]	TTAAACTAA	9	2	2	Secreted protease	477740–477748	NCTC11168	Variable in tandem repeat
V12	AAAAAAATT	9	2		Integral membrane protein	934077–934085	NCTC11168	Not tested further[g]
V13[b]	AAGAAAAAAAAATA	14	3.6	5	Noncoding	730103–730152	ICDCCJ07001[c]	Variable in tandem repeat
V14	TTCTATCATTTTTATCATC	18	3.1	4	Membrane protein, putative	1146381–1146435	ICDCCJ07001	Variable in tandem repeat[f]
V15	TAAAATTCACA	11	2.4	2	Rhomboid family protein	976742–976767	ICDCCJ07001	Invariable in tandem repeat[e]
V16	TTTTTGATAAAAT	13	2.3		Putative sugar transferase	1402318–1402347	ICDCCJ07001[c]	No amplified product in all test strains
V17[b]	TTTTGGGAT	9	3.4	2	Noncoding	651147–651177	81116	Variable in tandem repeat
V18	AGAATTTTTACT	12	2.8	0	Hypothetical protein	164129–164161	81116	Invariable in tandem repeat
V19[b]	AAAAAATAAAAAGAAAT	17	2.7	2	Noncoding	921640–921686	81116	Variable in tandem repeat
V20	ATTTTCTTTTGAT	13	2.6		Hypothetical protein	802080–802111	81116	No amplified product in most of test strains
V21	TATTTTAAAA	10	3.8		Noncoding	271296–271333	00-2544	Not tested further[f]

V22	ATTTTCTTTTGAT	13	2.6	0	Hypothetical protein	813683–813714	00-2544	Invariable in tandem repeat
V23	AAAAAAAGCTAGA	13	2.5	0	Noncoding	916910–916940	00-2544	Invariable in tandem repeat
V24	AAAAATTCACA	11	2.5		Amidohydrolase	589221–589248	00-2544	No amplified product in all test strains
V25	TTTTCTTTGATT	12	4.6	0	Hypothetical protein	796003–796057	81-176[c]	Invariable in tandem repeat
V26	AAAGAGTTAAAT	12	4.3	3	Hypothetical protein	71065–71115	81-176[c]	Invariable in tandem repeat[e]
V27	CAATTTTAACATTAT	15	6.5	0	Putative sugar transferase	1373487–1373584	IA3902	Invariable in tandem repeat
V28	CTTTTTATAAATATTAA	17	3.3		Noncoding	245643–245698	IA3902	No amplified product in most of test strains
V29	AAAATCTTGCG	11	2.7		Sugar transferase	1454132–1454161	00-2425[c]	No amplified product in all test strains
V30	TTTTAATAATATA	13	3.7	0	Copper-translocating P-type ATPase	1212484–1212535	RM1221[c]	Invariable in tandem repeat
V31	ATAAATAAAAAT	12	3.5		hypothetical protein	1066708–1066749	RM1221[c]	No amplified product in most of test strains
V32	TAGCAACAAA	10	3.2		Hypothetical protein	1039943–1039974	RM1221[c]	No amplified product in most of test strains
V33[b]	TTAAAAAAA	9	3.2	2	Rhomboid family protein	934591–934619	PT14[c]	Variable in tandem repeat
V34	ATTATTTTTAA	11	6.2		Noncoding	628827–628897	doylei 269.97[c]	No amplified product in all test strains
V35	TTTTCCTTTAAAAACAAAGCT	21	7.2		Hypothetical protein	655672–655822	S3[c]	No amplified product in most of test strains
V36	TATAATAATTAAAAG	15	3.7		Putative integral membrane protein	58087–58138	M1[c]	No amplified product in most of test strains

[a] Number of different fragment size polymorphisms detected among 10 *C. jejuni* isolates tested.
[b] Finally chosen for MLVA typing scheme.
[c] Uniquely found in that strain among 12 complete genomes of *C. jejuni* in GenBank database.
[d] Results are summarized under the results of different fragment size polymorphisms detected among 10 *C. jejuni* isolates tested.
[e] Found variable in PCR product size.
[f] Found variable in fragment sizes of the same number of TR, resulting in 4 fragment size variants. The sequencing data revealed that this locus was variable with 2 different patterns of TR.
[g] No appropriate primers were found.

TABLE 2 Primers and annealing temperature used for MLVA

Locus	Primer	Primer sequence (5′-3′)	Annealing temp (°C)
V2	V2F	CATCACTTCCTTGTTAAG	50
	V2R	CAATGTCCGTGATTATACA	
V6	V6F	GCAAGCTCATCAAGACTTT	55
	V6R	CTTTCYACCTCATTGCTATAA	
V11	V11F	ATGYCCTATGGTTCTACTTAG	55
	V11R	GCAGGCTTTGCCACT	
V13	V13F	TCAAGTAGAGTTTGTATTAGAACTTG	55
	V13R	TAACAATGTCCGTGATTATACA	
V17	V17F	CTCGTATTTATCCGCC	50
	V17R	TCATCTAACTCTTGACGC	
V19	V19F	TCCAAAAGGTTAAAAGCCT	55
	V19R	TGAAACGCATTATCTTACTATCTAG	
V33	V33F	TCAAACCAAGGATATTGTAATAAT	55
	V33R	CTGCTGATAATTTACCAAATGT	

(6, 25), VNTR loci of less than or equal to 20 bp in length, with copies numbers greater than or equal to 2 copies, were considered in this study. Using the Tandem Repeats Finder program revealed that most of the TR loci had repeat units with length greater than 10 bp and a small copy number (around two copies). The small number of the repeats that were available complicated the development of the MLVA assay. Up to 12 genomic sequences of the *C. jejuni* strains were used to search for the variable, polymorphic TR loci. Thirty-six different TR loci were selected and further tested for their polymorphism by using a set of 10 *C. jejuni* strains of 10 diverse origins (Table 1). Some repeat regions that were selected were common to several *C. jejuni* strains, while some selected regions were unique to a particular strain per the GenBank database. Finally, eight different TR loci (accounting for 22% of the tested TR loci), namely, V2, V6, V11, V13, V14, V17, V19, and V33, consistently yielded a band in the PCR and could be observed for some variation in the number of repeats among 9 out of 10 tested strains (Tables 1 and 3). Failure of amplification was detected in loci V6, V11, V14, V17, V19, and V33 of strain S9. Four out of the eight TR loci that were selected were located in noncoding regions. The other four were located in coding regions. Fifteen TR loci did not show variation in the number of repeats. Out of these 15 loci, V4, V15, and V26 loci showed variation in the size of the amplified products. Eleven VNTR loci could not be amplified for most of the strains that were tested. Loci V12 and V21 yielded multiple bands on multiple trials with different primers and under different conditions; therefore, they were excluded from further analysis.

Variability of VNTR loci in *C. jejuni* strains. Sequencing of the amplified PCR products showed that eight of the VNTR loci (V2, V6, V11, V13, V14, V17, V19, and V33) were polymorphic with five, two, two, five, two, two, two, and two different patterns in 10 *C. jejuni* isolates, respectively (Table 1). However, sequencing of PCR products of the locus V14 revealed that some variations in size of its PCR products were caused by flanking region sequences; there was the same number of TR (3 repeats) for amplicons of three different lengths, e.g., 323 bp in strain S14, 338 bp in strain T7, and 282 bp in strain ATCC 33560. Redesigning the primer pairs could not settle this issue. Moreover, different numbers of TR were found in two PCR products of the same size obtained from locus V14 (one repeat in a 288-bp amplicon in strain S12 and three repeats in a 282-bp amplicon in strain ATCC 33560). With this confounding data, fragment size analysis of V14 TR locus by CE, without DNA sequencing, would have led to misinterpretation of the results. Therefore, the V14 locus was excluded from MLVA. Considering the need for cost and time reduction and the accuracy of CE interpretation, this may allow laboratories not equipped with a DNA sequencer to perform the analysis, because the variation in the size of the fragment was confirmed to be the result of the variation of copy number of the repeats.

Based on sequencing data, locus V19 showed zero repeats in 4 of 10 *C. jejuni* isolates that were tested, possibly due to the absence of the corresponding locus in these isolates. However, locus V19 was the only locus that could successfully distinguish between *C. jejuni* strain S14 and T7. The six other VNTR loci that were selected in this study failed in this prospect. Therefore, locus V19

TABLE 3 MLVA patterns and DNA fragment lengths of 7 selected VNTR loci in a set of 10 different *C. jejuni* isolates[a]

	V2			V6			V11			V13			V17			V19			V33		
		bp			bp			bp			bp			bp			bp			bp	
Strain	TR	DNA	CE	TR	DNA	CE	TR	DNA	CE	TR	DNA	CE	TR	DNA	CE	TR	DNA	CE	TR	DNA	CE
S14	5	263	274	2	218	225	1	255	264	3.6	192	202	3.4	249	254	2	244	242	2	271	275
S12	5	265	275	2	218	225	2	264	274	3.6	192	201	4	259	267	2	244	242	2	271	276
K7	5	265	274	3	226	233	1	255	263	3.6	192	199	4	259	267	2.8	260	255	2	271	277
T9	5.9	277	287	2	218	224	1	255	263	4	205	209	4	259	270	0[b]	144	128	2	270	273
S6	6.5	283	294	2	218	225	1	255	262	4.8	211	220	4	262	269	2.8	260	255	2	270	273
T7	5	265	274	2	218	224	1	255	263	3.6	192	201	3.4	247	256	2.8	260	254	2	271	275
T10	5.9	275	285	2	219	225	1	256	264	4	201	213	3.4	250	256	0[b]	143	127	2	271	275
S9	11.5	344	352	—	—	—	—	—	—	7	271	275	—	—	—	—	—	—	—	—	—
ATCC33560	3	244	255	2	218	223	1	255	263	2	171	180	4	260	265	0[b]	143	126	2	271	276
JCM2013	5	267	275	2	218	223	1	257	263	3.6	192	202	4	259	265	0[b]	143	127	3	279	284

[a] TR, number of TRs; DNA, length (in bp) of DNA determined by DNA sequencing; CE, length (in bp) of DNA determined by CE; —, no amplification product was observed, even when different PCR primers and conditions were tried.
[b] Based on sequencing data, a repeat unit was absent.

TABLE 4 MLVA types of 50 *C. jejuni* isolates by MLVA with 7 VNTR loci

MLVA type	No. of repeats							No. of isolates	Isolate ID[a]	MLST sequence type(s)
	V2	V6	V11	V13	V17	V19	V33			
1	3	2	1	2	3.4	2.8	2	4	2, 9, 17, 18	4700
2	3	2	2	2	3.4	0[b]	2	2	31, 33	31
3	3	2	2	2	3.4	2	2	1	39	354
4	3	2	2	2	3.4	2.8	2	1	12	4358
5	3	2	2	3.6	3.4	2.8	2	1	4	624
6	3	2	2	3.6	4	1	2	1	35	4363
7	3	2	2	4.8	3.4	2.8	2	1	11	627
8	5	2	1	2	3.4	0[b]	2	2	(7), (8)	917, 1461
9	5	2	1	2	3.4	1	2	1	3	2439
10	5	2	1	3.6	3.4	0[b]	2	1	23	6720
11	5	2	1	3.6	3.4	1	2	4	41, 42, 45, 50	574
12	5	2	1	2	3.4	2	2	1	26	6720
13	5	2	1	3.6	3.4	2	2	2	(6), (25)	3765, 6720
14	5	2	1	3.6	3.4	2.8	2	1	22	6720
15	5	2	1	3.6	4	1	2	2	(5), (47)	1993, 574
16	5	2	1	3.6	4	2	2	1	1	1514
17	5	2	1	3.6	4	2.8	2	1	37	5722
18	5	2	1	7	3.4	1	2	1	10	1993
19	5	2	2	2	3.4	0[b]	2	1	24	773
20	5	2	2	2	3.4	2.8	2	1	27	347
21	5	2	2	3.6	3.4	0[b]	2	3	(28, 29), (36)	268, 536
22	5	2	2	3.6	3.4	2	2	2	32, 34	31
23	5	2	2	3.6	4	0[b]	2	1	30	268
24	5	2	2	4.8	3.4	0[b]	2	2	13, 14	187
25	5	3	1	3.6	4	1	2	1	44	574
26	6.5	2	1	3.6	3.4	1	2	1	15	1993
27	6.5	2	1	3.6	3.4	2.8	2	1	16	2433
28	6.5	2	1	4.8	3.4	2.8	2	6	19, 20, 40, 43, 48, 49	45
29	6.5	2	2	4.8	3.4	2.8	2	1	21	2751
30	6.5	3	1	4.8	3.4	1	2	1	38	583
31	6.5	3	1	4.8	3.4	2.8	2	1	46	45

[a] The isolate identifiers (ID) in different sets of parentheses are of different MLST sequence types [correlating to the different numbers in the "MLST sequence type(s)" column].
[b] Based on sequencing data, a repeat unit was absent.

was retained for further analysis by MLVA. Seven VNTR loci (V2, V6, V11, V13, V17, V19, and V33) finally were selected for MLVA. Ten different MLVA patterns (DI = 1.00) were generated based on the combinations of these loci that could successfully differentiate between the 10 *C. jejuni* isolates.

In this study, CE was used for the accurate estimation of the size of the PCR products for all loci. Fragment size obtained by CE did not exactly correspond to the actual fragment size identified by sequencing (2- to 11-bp difference) (Table 3). This could be due to the nature of the gel matrix, the slightly biased flanking sequences, or differences in mobility patterns of specific repeat units. The fragment size estimated by CE always shifts by a constant value (26, 27). However, this did not interfere with the overall results, as the number of repeats interpreted by sequencing or CE generated the same MLVA type in each isolate.

Stability of VNTR loci. In order to analyze the stability of the VNTR loci, two diverse strains of *C. jejuni* (strains S6 and ATCC 33560) were subcultured for 10 serial passages by streaking single colonies from each strain on CCDA plates. The plates were incubated from 24 to 48 h at 42°C in a microaerophilic atmosphere. MLVA pattern results obtained from the subcultured isolates were identical to those obtained from the original isolates (data not shown).

MLVA based on seven VNTR loci. MLVA was used to type a collection of 60 *C. jejuni* isolates obtained from chicken meat, chicken cecum, and environmental sources. The PCR products were previously analyzed by CE, and then the PCR products representing size variants were sequenced to confirm repeat copy numbers. The MLVA subtyping yielded a total of 39 MLVA types. Out of 39 MLVA types, 31 MLVA types (DI = 0.97) were detected in the 50 *C. jejuni* isolates used for comparisons with MLST. Locus V19 showed the highest diversity index (DI = 0.74), with four MLVA types, followed by loci V13 (DI = 0.67, 5 MLVA types) and V2 (DI = 0.61, 5 MLVA types). Low-diversity indices were detected in loci V11, V17, V33, and V6, which yielded 2 MLVA types by each of the loci and had diversity indices of 0.44, 0.35, 0.22, and 0.13, respectively.

Comparison of MLVA and MLST subtyping. To determine the value of MLVA for the molecular typing of *C. jejuni*, MLVA and MLST subtyping methods were compared using the results generated from 50 *C. jejuni* isolates. The results revealed that MLVA, with 7 VNTR loci, showed slightly higher differentiation of the *C. jejuni* isolates than MLST, yielding 31 MLVA types (DI = 0.97 with 21 MLVA types of a single strain) as opposed to 25 MLST sequence types (DI = 0.95 with 17 MLST sequence types of a single strain) (Table 4). The major advantages of MLVA over

MLST are its speed, simplicity in the processing and interpretation of the data, and lower costs (28), although the separation of the PCR products obtained in MLVA still requires capillary electrophoresis or an automated DNA sequencer to ensure accurate sizing of the PCR products. In our laboratory setting, the cost of MLVA (based on 7 VNTR loci) with CE per isolate was about 10 times lower than that of MLST (based on 7 housekeeping genes), while MLVA (based on 7 VNTR loci) with DNA sequencing was nearly the same cost as MLST (based on 7 housekeeping genes). The total analyzing time for MLVA with CE and DNA sequencing was about 8 to 9 h and 18 to 19 h per isolate, respectively, while the time for MLST was about 20 h per isolate.

To assess the congruence between typing methods, the adjusted Rand and Wallace coefficients were calculated. The overall congruence between MLVA and MLST, as determined by the adjusted Rand coefficient, was 63%, indicating moderate to good correlation between the two typing methods (29). The directional congruence, as estimated by Wallace coefficient going from MLVA to MLST, was 86%, suggesting that isolates assigned to a cluster by MLVA had a high probability of being assigned to the same cluster when typed by MLST. However, when examined in the other direction, there was a lower probability that isolates assigned to the same cluster by MLST (Wallace coefficient, 51%) would be assigned to the same cluster when typed by MLVA.

Although the results of MLVA were highly congruent with results obtained by MLST, there were differences in strain differentiation by different typing methods. This may be because of differences in the markers used for MLST (using housekeeping genes) and MLVA (using a set of diverse regions). Unlike MLST, MLVA uses various types of markers, such as genes involved in metabolism and genes associated with virulence (28). Among the 7 VNTR loci, four loci (V2, V13, V17, and V19) were located inside noncoding regions of the gene, while the other 3 loci (V6, V11, and V33) were located in coding regions. Locus V6 is located within the *ctsP* gene, which encodes an ATP/GTP-binding protein involved in cell proliferation, signal transduction, and protein synthesis. Locus V11 encodes a secreted protease involved in nutritional regulation, and locus V33 encodes a membrane protein which is a member of the rhomboid family of proteins.

In conclusion, the study describes the development of the MLVA method with seven novel VNTR loci to subtype *C. jejuni*. This method has slightly higher discriminatory power than MLST. The results of MLVA were congruent with results obtained by MLST, and MLVA predicted MLST type better than MLST predicted MLVA type. Although the MLVA method in this study might not replace MLST, MLVA could be used as a prescreening method in epidemiology before employment of MLST for analyzing a large population of *C. jejuni*. In the future, studies on additional VNTR loci and *C. jejuni* isolates can help to increase the discriminatory power of the method. Besides a comparison of MLVA with MLST, a comparison of MLVA with next-generation WGS, a recent typing method for *C. jejuni*, would be needed for future study.

ACKNOWLEDGMENTS

We thank Taradon Luangtongkum (Department of Veterinary Public Health, Chulalongkorn University) for donating the isolates of *C. jejuni*, Panvipa Phasipol for coordinating with the university and assisting in DNA extraction, Rabuesak Khumthong for advising the concordance coefficients, Chirapiphat Phraephaisarn for suggesting the laboratory techniques, and Satoko Miya for assisting with preparation of the manuscript.

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