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Multiple-locus variable-number tandem repeat analysis for strain typing of *Clostridium perfringens*

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Abstract

Clostridium perfringens is ubiquitous in the environment and causes diseases in man and animals, with syndromes ranging from enteritis, enterotoxemia, and sudden death to food poisoning and gas gangrene. Understanding the epidemiology of these infections and of the evolution of virulence in *C. perfringens* necessitate an efficient, time and cost effective strain typing method. Multiple-locus variable-number tandem repeat analysis (MLVA) has been applied to typing of other pathogens and we describe here the development of a MLVA scheme for *C. perfringens*. We characterized five variable tandem repeat (VNTR) loci, four of which are contained within protein encoding genes and screened 112 *C. perfringens* isolates to evaluate typability, reproducibility, and discriminatory power of the scheme. All the isolates were assigned a MLVA genotype and the technique has excellent reproducibility, with a numerical index of discrimination for the five VNTR loci of 0.995. Thus MLVA is an efficient tool for *C. perfringens* strain typing, and being PCR based makes it rapid, easy, and cost effective. In addition, it can be employed in epidemiological, ecological, and evolutionary investigations of the organism.

Keywords: MLVA; VNTR; *Clostridium perfringens*; Strain typing; Epidemiology

1. Introduction

Clostridium perfringens is ubiquitous in the environment and is part of the normal intestinal flora in man and animals [1–3]. Toxin types A-E are distinguished by their production of one or more so-called major toxins (alpha, beta, epsilon, and iota) [3]. All types of the organism are implicated in human and domestic animal diseases [3–13].

A useful method of strain typing would facilitate source tracking, development of strategies for prevention and control, and study of the organism's ecology [14] and evolution. Various techniques described include serotyping [15,16], bacteriocin typing [17–23], phage typing [24], plasmid profiling [20,25–27], multilocus enzyme electrophoresis (MLEE) [14,28], ribotyping [20,29], amplified fragment length polymorphism (AFLP) [30,31], and macrorestriction with pulsed field gel electrophoresis (PFGE) [29,32,33]. Many of these methods are highly discriminatory, but have insufficient typability, or are time- or cost-ineffective.

Multiple-locus variable-number tandem repeat analysis (MLVA) is more commonly used for strain typing of pathogenic microorganisms [34–50]. The method is based upon PCR amplification of variable tandem repeats (VNTRs), which are polymorphic DNA segments, from multiple genomic loci [51]. Molecular data generated by this method can be used for strain typing, population genetics studies [37,52–54] and as a source of the phylogenetic signal [37,38,45,54,55].

In this paper, we describe the development and application of a MLVA technique for *C. perfringens*.

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2. Materials and methods

2.1. Clostridium perfringens isolates and PCR template preparation

C. perfringens isolates (Table 1) were cultivated on brain heart infusion agar (Difco) supplemented with 5% bovine blood and 0.05% L-Cysteine, incubated overnight at 37 °C in an atmosphere of 80:20 H_2:CO_2. Several colonies suspended in 150 μL sterile HPLC-grade water were heated at 100 °C for 10 min. The mixture was clarified by centrifugation (13, 623*g*, 5 min) and 10 μL of 10-fold diluted (with sterile HPLC-grade water) supernatant was used as template for PCR reactions.

Evaluation of VNTR loci for polymorphism was performed initially on 10 strains (Table 1) and a further 102 strains were used to evaluate the technique. Isolates were from our collection, which consists mainly of North American field strains submitted for genotyping over a period of 15 years. The strains represent the five toxin types, irrespective of host species of origin, and ephemeral clones [54,56] likely to be of a single MLVA genotype were avoided when possible. Reproducibility of the technique was tested by screening 15 strains chosen at random.

2.2. Sequence search and primer(s) design

The *C. perfringens* ATCC 13124 unfinished genome sequence was obtained from The Institute for Genomic Research (TIGR) and that of *C. perfringens* plasmid (pCP13, GenBank accession no. AP003515) [57] from GenBank. Potential VNTR loci were identified using the standalone version of tandem repeat finder v 3.21 (TRF) [58] and tandem repeats database (version 2.09, http://tandem.bu.edu/cgi-bin/trdb/trdb.exe) was utilized to calculate and visualize the distribution of repeats. Parameters for repeat search included 2 matches, 3 mismatches, and 5 indels for pattern alignment, with 70 as the minimum alignment score and a maximum array size of 1000 bp. The plasmid sequence was searched according to the same parameters, but the minimum alignment score was 50.

Forward and reverse primers (Table 2) were designed using Primer3 software [59]. Choice of VNTR loci was by use of Nei's diversity index (D_i, otherwise known as polymorphism index) [41,44,60], and the number of loci required for the scheme was based upon the number of MLVA genotypes they resolved and on the value of the numerical index of discrimination for the method (D) [14,61]. MLVA loci (Table 3) were designated as previously described [40,44].

BLASTX and BLASTN algorithms were used for database similarity search [62,63] of the *C. perfringens* strain 13 genomic sequence and of other bacterial sequences in the NCBI database, using the default parameters with the low complexity filter turned-off.

2.3. PCR and VNTR analysis

Each 50 μL PCR reaction mixture contained 50 pmol of each primer (Sigma Genosys) and 5 U of *Taq* DNA polymerase in storage buffer A (Promega). The supplied *Taq* buffer and the four dNTPs were added to a final concentration of 1X and of 0.2 mM, respectively. CP6 and CP13 were multiplexed in one reaction while the remaining loci were done individually. PCR amplification consisted of a hot start (95 °C, 3 min), followed by 35 cycles of denaturing (95 °C, 1 min), annealing (50 °C, 1 min), extension (72 °C, 1 min), and a final extension (72 °C, 5 min). PCR products were separated by electrophoresis in a 3% (wt/vol) agarose gel (GENE-Mate) in TAE buffer at 5 V cm^{-1}, with size standards (100 bp DNA ladder, New England Biolabs) in every fourth lane (Fig. 1). Gels were visualized by UV transillumination, and photographs were digitized. Positive (JGS 1842) and negative (water) controls were run with each experiment. All negative occurrences for each VNTR locus were repeated after 2 months to exclude any human or pipetting errors.

2.4. Data analysis

Digital images were imported into GelCompar II software v 3.5 (Applied Maths). Band matching was initially performed with arbitrary values for optimization (1%) and position tolerance (1%), and calculation of optimal values for these parameters, in each of the four VNTR experiments, was based upon the characteristics of resulting major clusters. VNTRs data were concatenated into a single character table and each band class of a unique molecular size represented an allele. The similarity matrix was calculated using Dice coefficient [64] and the phylogenetic tree was generated using the neighbor-joining (NJ) algorithm [65]. The robustness of the tree and tree clusters was tested by cophenetic correlation implemented in GelCompar software. *Clostridium difficile* JGS 370 was used as an out-group to predict the root of the tree.

3. Results and discussion

3.1. Identification of polymorphic VNTR loci

VNTRs are essentially minisatellites, and though many are polymorphic, others do not show any variation within a population [41]. Le Flèche et al. (2001) found significant correlation between minisatellite polymorphism and both the array length and GC

Table 1
C. perfringens isolates used in this study

JGS #	Genotype	Species of origin	Disease
JGS 1501	A	Avian	Necrotic enteritis
JGS 1521	A	Avian	Necrotic enteritis
JGS 1620	A	Avian	Unavailable
JGS 1657[a]	A	Avian/chicken	Unavailable
JGS 1677	A	Avian	Unavailable
JGS 4042	A	Avian/western Bluebird	Necrotic enteritis
JGS 4066	A	Avian/chicken	Necrotic enteritis
JGS 4059[a]	A	Avian/chicken	Necrotic enteritis
JGS 1004	AE	Human	Unavailable
JGS 1292	AE	Human	Unavailable
JGS 1612	A	Human	Feces
JGS 1711	AE	Human	Unavailable
JGS 1719	AE	Human	Unavailable
JGS 1313	A	Canine	Intestine/small
JGS 1320	A	Canine	Reproductive tract
JGS 1338	A	Canine	Conjunctiva
JGS 1357	A	Canine	Chronic colitis
JGS 1363	A	Canine	Enteritis
JGS 1413[a]	AE	Canine	Enteritis
JGS 1414[a]	AE	Canine	Enteritis
JGS 4032	AE	Canine	Unavailable
JGS 1533[a]	A	Bovine	Unavailable
JGS 1693	A	Bovine	Enteritis
JGS 1703	A	Bovine	Enteritis
JGS 1736	A	Bovine	Enteritis
JGS 1742	A	Bovine	Enteritis
JGS 1771	A	Bovine	Enteritis
JGS 1796	A	Bovine	Enteritis/diarrhea
JGS 1825	A	Bovine	Enteritis/diarrhea
JGS 1826	A	Bovine	Enteritis/diarrhea
JGS 1853	A	Bovine	Sudden death
JGS 1869[a]	A	Bovine	Enteritis
JGS 1892	A	Bovine	Sudden death
JGS 4054[a]	A	Bovine	Unavailable
JGS 4080[a]	A	Bovine	Septicemia/toxemia
JGS 1013	A	Cervidae/deer	Unavailable
JGS 1243	A	Cervidae/white tailed deer	Lactic acidosis
JGS 1323	A	Cervidae/deer	Caecum
JGS 1549	A	Cervidae/elk	Unavailable
JGS 1622	A	Cervidae	Unavailable
JGS 1665	A	Cervidae/deer	Hemorrhagic enteritis
JGS 1739	A	Cervidae/deer	Unavailable
JGS 1791	A	Cervidae/deer	Unavailable
JGS 1805	A	Cervidae	Unavailable
JGS 4006	A	Cervidae	Unavailable
JGS 4119	A	Cervidae/Caribou	Diarrhea
JGS 1842[a]	A	Equine	Hemorrhagic colitis
JGS 4099[a]	A	Equine	Diarrhea
JGS 4151	A (strain 13)	—	—
JGS 4175	A (ATCC13124)	—	—
JGS 1118	B	Ovine	Unavailable
JGS 1984	B	Unavailable	Unavailable
JGS 1015	C	Bovine	Unavailable
JGS 1022	CE	Canine/greyhound	Food
JGS 1070	C	Porcine	Enteritis
JGS 1076	C	Porcine	Enteritis
JGS 1090	C	Porcine	Unavailable
JGS 1142	C	Bovine	Acute enteritis
JGS 1164	C	Bovine	Enteritis
JGS 1460	C	Porcine	Unavailable
JGS 1475	C	Porcine	Unavailable
JGS 1495	C	Porcine	Unavailable

Table 1 (*continued*)

JGS #	Genotype	Species of origin	Disease
JGS 1504	C	Porcine	Necrotic enteritis
JGS 1527	C	Porcine	Unavailable
JGS 1543	C	Porcine	Unavailable
JGS 1544	C	Porcine	Unavailable
JGS 1564	C	Porcine	Unavailable
JGS 1565	C	Equine	Unavailable
JGS 1640	C	Porcine	Unavailable
JGS 1659	C	Porcine	Enteritis/diarrhea
JGS 1696	C	Unavailable	Unavailable
JGS 1706	C	Unavailable	Unavailable
JGS 1182	D	Ovine	Sudden death
JGS 1240	D	Ovine	Bronchopneumonia
JGS 1458	D	Ovine	Unavailable
JGS 1551	D	Ovine	Unavailable
JGS 1558	D	Caprine	Diarrhea
JGS 1705	D	Ovine	Unavailable
JGS 1721	D	Ovine	Enteritis
JGS 1768	D	Unavailable	Unavailable
JGS 1841	D	Unavailable	Unavailable
JGS 1902	DE	Ovine	Enteritis
JGS 1927	D	Seed Culture	Unavailable
JGS 1941	D	Caprine	Unavailable
JGS 1942	D	Caprine	Sudden death
JGS 1944	D	Caprine	Enterotoxemia
JGS 1945	D	Caprine	Diarrhea
JGS 4105	D	Ovine	Sudden death
JGS 4117	D	Unavailable	Unavailable
JGS 4138	DE	Caprine	Sudden death
JGS 4152	DE	Ovine	Pulpy kidney disease
JGS 4158	DE	Caprine	Enteritis/septicemia
JGS 1478	EE	Bovine	Enteritis
JGS 1482	EE	Bovine	Enteritis
JGS 1496	EE	Bovine	Enteritis
JGS 1499	EE	Bovine	Enteritis
JGS 1506	EE	Bovine	Enteritis
JGS 1510	EE	Bovine	Enteritis
JGS 1511	EE	Bovine	Enteritis
JGS 1547	EE	Bovine	Enteritis
JGS 1553	EE	Bovine	Enteritis
JGS 1792	EE	Bovine	Enteritis
JGS 1884	EE	Bovine	Enteritis
JGS 1901	EE	Bovine	Enteritis
JGS 1903	EE	Bovine	Enteritis
JGS 1943	EE	Bovine	Enteritis
JGS 1975	EE	Bovine	Enteritis
JGS 1985	EE	Bovine	Enteritis
JGS 1986	EE	Bovine	Enteritis
JGS 1987	EE	Bovine	Enteritis
JGS 4071	EE	Bovine	Enteritis
JGS 4154	EE	Bovine	Enteritis

[a]Isolates used for initial screening of VNTR loci.

content in *B. anthracis*, while examination of *Y. pestis* revealed strong correlation with homogeneity of repeats. However, no algorithm can yet predict minisatellite polymorphism efficiently in bacterial sequences [66]. We chose repeat search parameters to reflect some of the above-mentioned correlations and we identified 500 direct repetitive sequences in the chromosome of *C. perfringens* ATCC 13124, most of which (96%) have a pattern size of 10–100 bp. About three-quarters of the loci had <10 repeat copies and about 20% were $\geqslant 40\%$ G + C. Array length $\leqslant$ 100bp was found in ~47% of the repeat loci. Of 78 repeat loci identified in the plasmid, ~77% had <10 repeat copies, and ~97% contained $<40\%$ G + C. The pattern size was 10–100 bp for ~80% of the loci, and 51% were <100 bp in array length.

Table 2
Primers for amplification of VNTR loci

VNTR locus	Primers	T_m (°C)	T_a (°C)
CP6_2131_18 bp_28.2 U	CP6-F 5′ GTAAAGATGATTGCTATTTAGAGATAA	54	50
	CP6-R 5′ TAAGGTATCATCAAAATCCACTCCAGG	64	
CP13_1813_9 bp_16.3 U	CP13-F 5′ AAGGAAGATGCTACTCAAGATG	55	50
	CP13-R 5′ GAAGCCATCATTACTACTCCTA	53	
CP16_2_6 bp_68 U	CP16-F 5′ AAAGTTCCAGGTAAAATAAGAG	51	50
	CP16-R 5′ CATTCTCTTTCATTCTCTGTAA	51	
CP19_2339_20 bp_24.9 U	CP19-F 5′ CTCAATCCTAACAATATGTGCTGACTA	59	50
	CP19-R 5′ GTAGCAGCAATAAAACCAACCTAAA	60	
CP42_2843_21 bp_20 U	CP42-F 5′ GATGGCCCAAGAAACAGAAC	59	50
	CP42-R 5′ GCTGGGAATAAAGGGTTTGA	59	

T_m: melting temperature; T_a: annealing temperature.

Table 3
Characteristics of tandem repeat loci

VNTR locus	Pattern length (bp)	Repeat copy number	Expected band size (bp) [a]	% GC	% matches	Location	Number of alleles[b]	D_i
CP6_2131_18 bp_28.2 U	18	28.2	601	56	76	Chromosomal[c]	8	0.695
CP13_1813_9 bp_16.3 U	9	16.3	295	43	73	Chromosomal	7	0.753
CP16_2_6 bp_68 U	6	68	1061	20	66	Episomal	7	0.743
CP19_2339_20 bp_24.9 U	20	24.9	1009	24	71	Chromosomal	16	0.872
CP42_2843_21 bp_20 U	21	20	1338	32	69	Chromosomal	13	0.828

D_i was calculated as $1 - \Sigma$ (allele frequency)^2. The value of D_i based on the 112 isolates analyzed in this study.
[a]According to the source sequence.
[b]Based on screening of 112 *C. perfringens* isolates and including the null allele.
[c]No null allele was detected at this locus.

Selection of candidate loci was based on repeat pattern sizes and copy numbers suitable for detection by agarose gel electrophoresis. We screened 42 loci in a group of 10 strains (Table 1) and those with at least two alleles (including null allele) were considered for further analysis. Five loci had values >0.5 in Nei's diversity index, meaning that there is $>50\%$ probability that two alleles chosen at random are different from each other (Table 3), while others were excluded due to poor amplification or low diversity index values.

3.2. MLVA scheme for Clostridium perfringens

The number of VNTR loci needed to discriminate among isolates was based in part upon the number of MLVA genotypes resolved by addition of loci to the analysis and a plateau in this relationship was used as a cut-off. In addition, the numerical index of discrimination of the combined loci was set at $\geqslant 0.9$, as previously suggested [14,61].

A plateau can be reached when each strain is assigned to a MLVA genotype but clones containing two or more strains forestall that. This is probably not uncommon, since *C. perfringens* spores are considerably resistant in the environment [67–70] and may be mechanically transported form one locality to another. This suggests that two strains might match even if recovered from different geographical locations and/or at different times. In these studies, there is a direct relationship between the number of loci examined

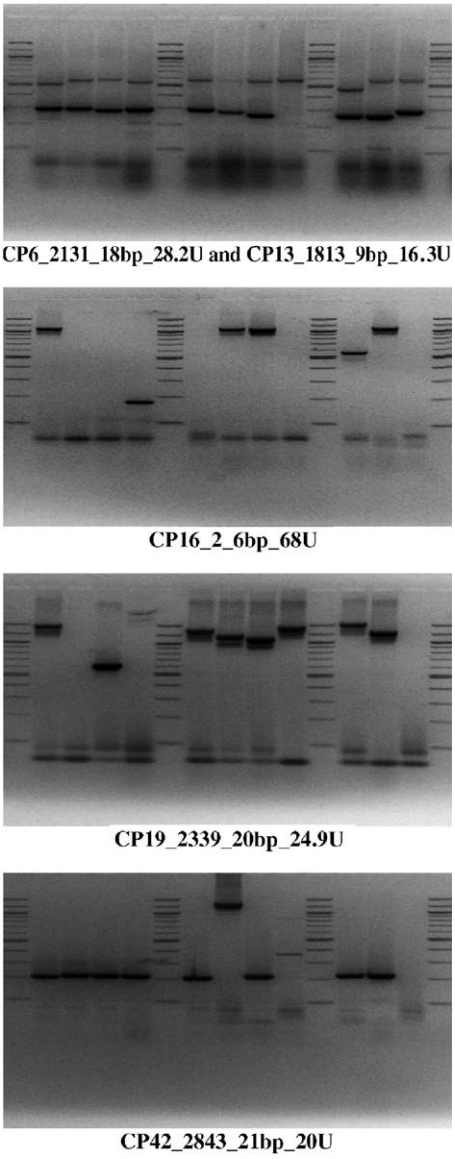

Fig. 1. VNTR analysis in 3% (wt/vol) agrose electrophoresis. Lanes 1, 6, 11 and 15 are 100 bp DNA ladder. Isolates form left to right are JGS 1984 (Lane 2, unpublished data), JGS 1413 (lane 3), JGS 1414 (lane 4), JGS 1533 (lane 5), JGS 1657 (lane 7), JGS 1842 (lane 8), JGS 1869 (lane 9), JGS 4054 (lane 10), JGS 4059 (lane 12), JGS 4080 (lane 13), and JGS 4099 (lane 14).

and the number of MLVA genotypes resolved (Fig. 2), with an exponential increase up to the fourth locus. Examination of clones detected on the tree (Fig. 4) revealed that three [(JGS 1070; JGS 1076), (JGS 1015; JGS 1022), and (JGS 1711; JGS1719)] are likely to be true clones based on epidemiological data. The numerical index of discrimination reached a value of 0.995 when five loci were included in the analysis (Fig. 3). Thus, five loci are likely sufficient for MLVA analysis of *C. perfringens*.

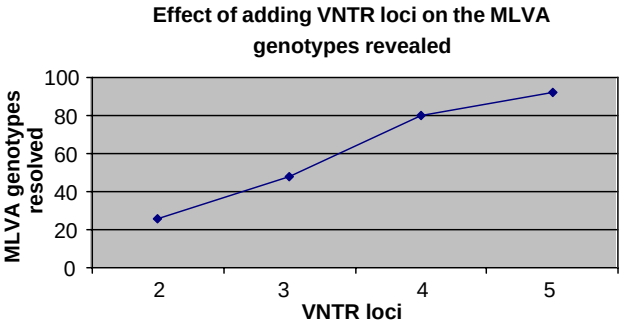

Fig. 2. Effect of adding VNTR loci on the MLVA genotypes resolved.

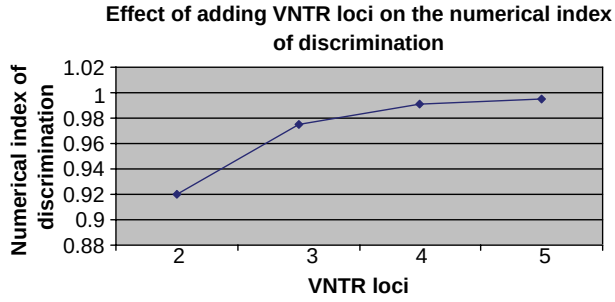

Fig. 3. Effect of adding VNTR loci on the numerical index of discrimination.

3.3. Characteristics of the *C. perfringens* MLVA loci

3.3.1. CP6_2131_18 bp_28.2 U

The repeat locus of 507 bp mapped to the collagen—like protein of strain 13 (CPE0955), and sequence amplified by the forward and the reverse primer lies within the open reading frame of CPE0955, as well. The source sequence has 97% identity over the 203 amino acid overlap and the most common allele is ~570 bp.

3.3.2. CP13_1813_9 bp_16.3 U

The expected DNA band mapped to *nagK* (CPE1279). The source sequence has 85% identity and 90% similarity over the 97 amino acid overlap. In strain 13, five genes (*nagHIJKL*) involved in hyaluronidase production are widely separated on the chromosome [57]. In addition, *nagK* has 6 alleles in the population (not including the null allele), and the most common is ~282 bp. This poses the question of the importance of hyaluronidase to *C. perfringens*, and whether alleles at the *nagK* locus, that are represented frequently in the population, share in the adaptation of this organism to its ecological niche. Further sampling of the population, and functional as well as evolutionary analysis of these gene(s) product(s) are required to answer this question.

3.3.3. CP16_2_6 bp_68 U

The repeat locus of 433 bp is located within the open reading frame of pCP13 *parB* (PCP02). However,

forward and the reverse primers amplify 276 bp upstream and 352 bp downstream of the repeat locus, respectively. The 276 bp upstream as well as the 100 bp downstream of the locus are located within *parB* open reading frame, and the 5′ end of the reverse primer binds within the open reading frame of a hypothetical protein, PCP03, ~249 bp downstream from the 3′ end of *parB*. The nearest direct repetitive sequence that could be located using different search parameters lies approximately 1940 bp downstream from the 3′ end of *parB*, suggesting that the band size polymorphism is likely to be a function of the direct repeats located within *parB*. The most common allele is ~1058 bp.

3.3.4. CP19_2339_20 bp_24.9 U

The repeat locus of 481 bp showed no significant similarity on the protein level with other sequences at the NCBI database. Of the 480 bp amplified by the forward primer, 363 or 387 bp mapped to the probable ferrous iron transport protein B gene (CPE1660) of strain 13 or *C. tetani* E88 ferrous iron transport protein B (CTC00451), respectively. The source sequence has 21% identity and 44% similarity over the 122 amino acid overlap, compared to strain 13, and 67% identity and 84% similarity over the 129 amino acid overlap, compared to *C. tetani*. Repeats were not detected in the upstream or downstream sequences amplified by the primer set, suggesting that polymorphism in band size is likely due to rounds of expansion or shrinkage of the repeat locus. The null allele is the most common at this locus.

3.3.5. CP42_2843_21 bp_20 U

The primer set amplifies 460 bp upstream and 476 bp downstream from the repeat locus. Forty-two basepairs, including the forward primer sequence, mapped to a strain 13 conserved hypothetical protein (CPE0571), and the source sequence has 100% identity over the 14 amino acid overlap. In addition, 66 bp including the reverse primer sequence mapped to the riboflavin synthase beta subunit (CPE0569) of strain 13, and the source sequence has 100% identity over the 22 amino acid overlap. On the other hand, the repeat locus of 402 bp, 310 bp upstream, and 278 bp downstream mapped to the open reading frame of hypothetical protein CPE0570 of strain 13. The source sequence has 86% identity and 87% similarity over the 330 amino acid overlap. The sequence upstream and downstream from the repeat locus did not reveal any direct repeats suggesting that variation in band size is likely to be a function of the repeats within CPE0570. The most common allele is ~200 bp.

3.4. MLVA for C. perfringens strain typing

Strain typing is essential for understanding the epidemiology of *C. perfringens* infections, as well as the ecology and evolution of the organism. MLVA is increasingly in use for strain typing of pathogenic organisms, and it has proven to have excellent typability, discriminatory power, medium cost, ease of performance, and accessibility [36,43,44,46,47,71,72]. Application of the method allowed assignment of each isolate to a MLVA genotype; no strain had null alleles for all five loci. Strains that match in the five loci were assigned to the same genotype (or clone). CP13 and CP42 were null alleles in 3.6% of the strains while the CP16 locus was a null in ~25% of the total number of strains.

When human and pipetting errors are ruled out, successful PCR amplification depends essentially on the degree of identity between the primer sequence and target sequence. Even in protein-encoding genes that are under purifying selection, synonymous substitutions would lead to a decrease of this identity within the organism population. This suggests that it is probable to end with poor amplification and a weak signal (very faint band) when running VNTR analysis, and in this case the amplification process would be expected to happen by chance. In testing reproducibility, all MLVA loci data for the 15 isolates were reproducible, and weak signals did not exceed 1% (4 occasions out of 560 for 112 isolates tested). In addition, the Dice coefficient used to calculate genetic distance do not consider negative co-occurrence as a proof of genetic similarity [73]. This suggests that it is unlikely that two phylogenetically distant isolates will be considered similar to one another.

The MLVA method based upon five loci has a discrimination index of 0.995, meaning that if two isolates are sampled randomly of the population, they will belong to different MLVA genotypes on 99.5% of occasions. This discrimination power is comparable to that of esterase electrophoretic typing and slightly higher than that of MLEE, described previously [14,28].

Long-term epidemiological value of any bacterial typing methods relies not only on their ability to discriminate between isolates, but also on the probability of detecting the same allele(s) over time [40]. Isolates included in the study were chosen randomly with respect of genotyping date and our collection is constructed over a period of 15 years. There is approximately 20–40% probability of detecting the same allele as calculated by Nei's diversity index. Moreover, clones were detected on the tree and some of them have isolates that show temporal differences, indicating the suitability of the MLVA scheme of epidemiological studies.

Unweighted pair-group method with arithmetic mean (UPGMA) is the most frequently used method for tree

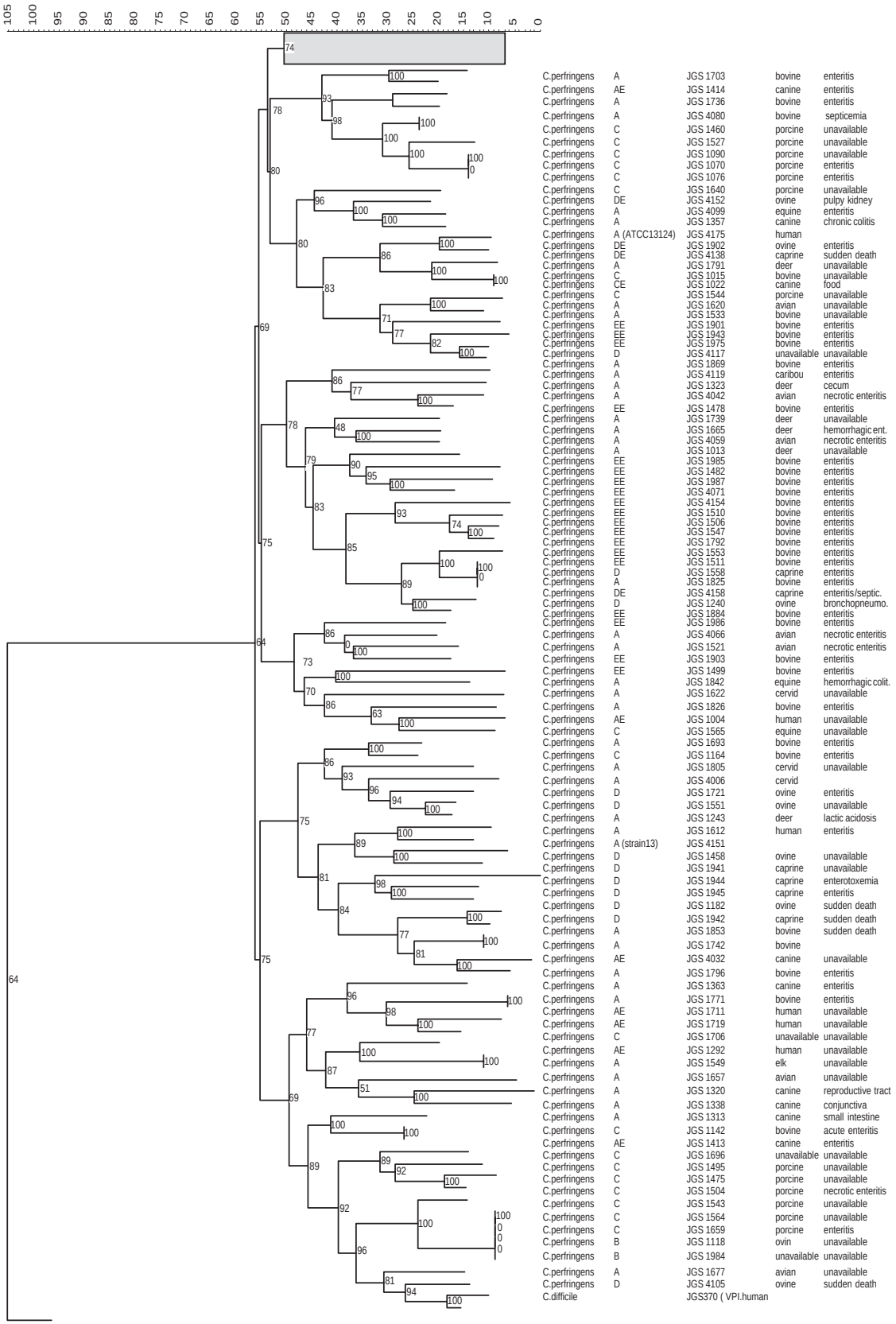

Fig. 4. NJ tree generated from MLVA data using Dice coefficient. The scale on the top of the figure is genetic distance and the tree is additive meaning the branches are not equidistant from the root. The distance between two operational taxonomic units, OTUs (strains) equal the sum of lengths of all the branches connecting them [75].

construction from MLVA data [35,36,38,42,43,46,74]. However, computer simulation studies showed that the UPGMA is inferior to the NJ method for phylogeny inference [75]. We used the NJ method to describe the genetic distances among the tested isolates, since it was not our intention to draw phylogenetic inference from the MLVA data in this paper; this necessitates a better representation of the population. The additive tree (Fig. 4) of the tested isolates shows two major clades with isolates displaying various genetic distances from one another. All type EE isolates are in one major clade, and all clones are formed by isolates that belong to a single major toxin type. Interesting enough, in many cases type A isolates are phylogenetically close to isolates from other major toxin types. Almost all type A isolates screened in this work are recovered from disease conditions.

Finally, this technique shows all the necessary characteristics of an efficient strain typing method. Its PCR basis should ensure ease of transfer and application across laboratories and rapid acquisition of results. We plan to continue sampling of *C. perfringens* population(s) and testing different hypotheses using phylogeny to shed light on the evolution of this organism.

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