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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