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Comparison of 2 proposed MLVA protocols for subtyping non-O157:H7 verotoxigenic *Escherichia coli*☆

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ABSTRACT

Multiple *locus* variable number tandem repeats (VNTRs) analysis (MLVA) has become a reliable tool, able to establish genetic relationships for epidemiological surveillance and molecular subtyping of pathogens such as verotoxigenic *Escherichia coli* (VTEC). This emerging pathogen whose primary reservoir is the cattle causes severe disease in humans, such as hemorrhagic colitis and hemolytic uremic syndrome. With the aim of comparing a recently proposed MLVA assay with that used routinely in our laboratory, we analyzed a set of VTEC isolates (n = 72) obtained from meat using both assays. All samples could be typed by the new MLVA assay, and an increase in the number of distinct profiles (31–43) was observed. However, intraserotype resolution was not significantly enhanced; thus, the incorporation of more VNTR loci is still needed to achieve a greater discrimination among non-O157:H7 serotypes.

1. Introduction

Variable number tandem repeats (VNTRs) comprise variable regions of many of the bacterial genomes. They consist of short sequences (known as repeat units), which are repeated in tandem, and vary in the number of copies, creating length polymorphisms. Multiple *locus* VNTR analysis (MLVA) is a reliable tool, able to establish genetic relationships between bacterial strains for epidemiological surveillance and molecular subtyping of pathogens such as verotoxigenic *Escherichia coli* (VTEC) (Keys et al., 2005; Lindstedt et al., 2003, 2004, 2007; Noller et al., 2003, 2004, 2006).

VTEC has been identified as an important causal agent of hemolytic uremic syndrome (HUS) and bloody diarrhea (Griffin, 1998), being a frequently cause of gastrointestinal disease in America and Europe (Beutin et al., 1998; Huppertz et al., 1996; Johnson et al., 2006). Cattle colonized by VTEC are thought to be the primary reservoir of this bacterium, and its transmission to humans frequently results from the ingestion of contaminated food of bovine origin (Karmali, 1989; Nataro and Kaper, 1998). The VTEC group is very diverse being O157:H7 the most common serotype associated with sporadic cases and large outbreaks of HUS in many countries (Armstrong et al., 1996; Tarr, 1995). However, there is a growing concern about the risk to human health associated with non-O157 VTEC serotypes (Johnson et al., 2006).

MLVA allows differentiating closely related isolates and, at epidemiological level, source tracking during investigation of outbreaks, as well as the interpretation of suspected outbreaks. Several MLVA protocols have been developed for the specific typing of VTEC O157:H7 (Keim et al., 2000; Lindstedt et al., 2004; Noller et al., 2003) and other ones for non-O157:H7 serotypes (Izumiya et al., 2010; Lindstedt et al., 2007; Manges et al., 2009).

In previous studies, we implemented a generic *E. coli* MLVA assay based on the combination of 7 loci with different degrees of polymorphism proposed by Lindstedt et al. (2007), called by us $MLVA_{G7}$. The results confirmed the usefulness of this assay for the genetic characterization of native VTEC strains of different serotypes but pointed out the need to incorporate new repeat-containing loci for the characterization of non-O157 isolates (Bustamante et al., 2010; Franci et al., 2011). Recently, Løbersli et al. (2012) extended the previously published $MLVA_{G7}$, incorporating 3 more loci, 2 VNTRs (CVN016, CVN017), and a CRISPR locus (CCR001). In the present study, this extended protocol is designed as $MLVA_{G10}$.

The clustered, regularly interspaced, short palindromic repeat (CRISPR) loci are a class of DNA sequences identified by Jansen et al. (2002) characterized by direct repeats of 24–47 base pairs (bp), separated by sequences of variable size, of between 21 and 72 bp, called spacers (Grissa et al., 2007; Horvath and Barrangou, 2010). Such sequences originally called short regularly spaced repeats have been detected in different prokaryotes and have a similar structure in phylogenetically distant groups (Mojica et al., 2000).

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The importance of tracking outbreaks of foodborne illness and the emergence of new virulent subtypes of foodborne pathogens have created the need for rapid and reliable subtyping methods for VTEC. In Argentina, 40% of cases of infection with VTEC serotypes are due to non-O157:H7 (Rivas et al., 2008), and this trend worldwide is also increasing (Coombes et al., 2008), so it is important to have a fast methodology for the study of genetic polymorphisms of any VTEC serotype. The objective of this study was to evaluate the power of discrimination of the new set of markers for typing VTEC strains isolated from meat products, most of which have been previously analyzed by $MLVA_{G7}$ (Bustamante et al., 2010; Franci et al., 2011).

2. Materials and methods

2.1. Bacterial strains

A total of 72 isolates from the collection of the Laboratorio de Inmunoquímica y Biotecnología (UNCPBA, Tandil, Argentina) were studied. They had been collected between 1998 and 2003 from minced meat, hamburgers, and viscera (Parma et al., 2000; Sanz et al., 2007). The isolates belonged to 25 serotypes: O2:NM (1), O8:H16 (2), O8:H19 (9), O20:H19 (2), O22:H8 (2), O79:H19 (2), O88:H21 (1), O91:H21 (1), O112:H2 (1), O113:H21 (3), O113:NM (10), O116:H21 (1), O117:H7 (4), O157:H7 (1), O171:H2 (1), O171:HNT (1), O171:NM (2), O174:H21 (6), O178:H19 (5), O185:H7 (1), ONT:H7 (5), ONT:H8 (1), ONT:H19 (2), ONT:H21 (4), and ONT:HNT (4). They have been previously analysed by PCR for the presence of genes encoding for verocytotoxin 1 and 2 (*vtx1* and *vtx2*), intimin (*eae*), enterohaemolysin (*ehxA*), STEC autoagglutinating adhesin (*saa*) (Blanco et al., 2004; Lucchesi et al., 2006; Parma et al., 2000; Sanz et al., 2007).

2.2. MLVA typing

The 10 VNTR loci studied, and the primers used were those proposed by Lindstedt et al. (2007) and Løbersli et al. (2012). The 7 loci proposed by Lindstedt et al. (2007) were analyzed, as described by Bustamante et al. (2010). Sixty isolates have been previously subtyped by $MLVA_{G7}$ in our laboratory (Bustamante et al., 2010; Franci et al., 2011). The 3 additional loci proposed by Løbersli et al. (2012), CVN016, CVN017 and CCR001, were amplified using monoplex PCR assays. Each PCR was carried out in a 25-μL volume containing 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 9, 0.1% Triton X-100), 2.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 2.5 pmol of each primer with 0.5 U Taq DNA polymerase (InBio, Highway, Argentina) and 2 μL of crude DNA extract. Thermal cycling conditions were the same as those detailed by Løbersli et al. (2012). PCR products were run in 3% agarose gels stained with SYBR Safe (Invitrogen, Inc. Carlsbad, CA, USA).

Allelic variants identified for each VNTR were sequenced with the same primers used to amplify those regions, using an ABI PRISM 3730XL analyzer (Macrogen, Inc, Seoul, Korea). The sequences obtained were analyzed using the software Chromas 2.32 (Technelysium Pty. Ltd., Tewantin, Australia) and those corresponding to the alleles of each VNTR aligned with the software Clustal W (Larkin et al., 2007).

2.3. Data analysis

The alleles were named according to the number of tandem repeat units (RU). The absence of amplification product was considered null allele, and it was designed with the arbitrary number 30. Cases in which an amplification product was observed but the repeated region was absent in the sequenced region were encoded with the arbitrary number 40. Alleles that showed partial repeats were designed with the closest complete repeat number (Hyytiä-Trees et al., 2006).

The diversity index (D_N) was calculated for each locus using the formula $D_N = 1 - \sum(fr_a)2$, where fr_a is the allelic frequency (Noller et al., 2003). MLVA profiles were defined either taking into account the alleles at each of the 7 loci proposed by Lindstedt et al. (2007)—$MLVA_{G7}$—or including also the alleles for the 3 loci proposed by Løbersli et al. (2012)—$MLVA_{G10}$. Dendrograms were constructed using UPGMA cluster analysis implemented by software START Vs. 1.0.5 (Joley et al., 2001). The discrimination power of each of the methods (for the same set of samples) was assessed using the Simpson diversity index (D_S) (Hunter and Gaston, 1988).

In order to compare the proportion of isolates showing unique MLVA profiles identified by both methods, the McNemar test was used. Also, to estimate the concordance between $MLVA_{G7}$ and $MLVA_{G10}$ to detect unique MLVA profiles, we carried out an analysis using FREQ procedure of SAS v9.13 (Cary, NC).

3. Results and discussion

In this study, VTEC isolates belonging to 25 serotypes were characterized by 2 MLVA assays, one based on 7 loci ($MLVA_{G7}$) and another one including 3 more loci ($MLVA_{G10}$), in order to compare these approaches. For 60 isolates, CVN016, CVN017, and CCR001 data were added to the $MLVA_{G7}$ data panel, previously obtained by Bustamante et al. (2010) and Franci et al. (2011). For the remaining 12 isolates, which had not been previously subtyped by MLVA, the 10 VNTR loci were amplified. Regarding these 12 isolates analyzed for the first time by $MLVA_{G7}$, new alleles (with respect to previously detected in our laboratory) for 2 loci were found, allele 4 for locus CVN001 and alleles 2 and 13 for CVN014. CVN003 locus was absent in 100% of the non-O157 isolates, agreeing with our previous results (Franci et al., 2011). Lindstedt et al. (2007), Løbersli et al. (2012), and Naseer et al., (2012) also observed null alleles for this locus in the majority of the isolates.

Regarding the 3 loci incorporated into the MLVA panel, CVN016 locus showed the highest variability with 6 alleles (Table 1), and a new allele variant with 3 RU was detected, in comparison to previous publications (Løbersli et al., 2012; Naseer et al., 2012). Only 2 isolates showed null alleles for this locus.

All the isolates showed null alleles for the locus CVN017, suggesting either locus absence or sequence polymorphism at priming sites. Løbersli et al. (2012) also observed null alleles for this locus in most of their samples, except in those belonging to serogroups O26, O103, O121, O145, and O177, while Naseer et al., (2012) omitted this locus in their study.

The CCR001 locus showed an RU of about 26 bp, a spacer of about 34 bp, and alleles with 2 or 3 RU, both included within the range detected by Løbersli et al. (2012). Allele 2 was the most commonly found; meanwhile, the allele with 3 RU was mainly present in isolates belonging to O8:H19 serotype. Sequencing of PCR products showed, on occasions, the same number of RU for amplimers of different lengths. On the other hand, the 2 O8:H16 isolates showed amplification products at this locus (data not shown); however, when we analyzed the sequences, we did not find the repeat region. This allele was arbitrarily designated with the number 40. Naseer et al., (2012) reported a remarkably broad allelic range for this locus (6–95), but the finding of alleles with such a high number of repetitions for a CRISPR locus is uncommon.

$MLVA_{G10}$ data showed a number of alleles detected per locus ranging from 1 (CVN003) to 13 (CVN014), except the locus CVN017, which did not amplify in any of the samples. The number of repeats identified among the 10 loci analyzed ranged from 1 to 20, and a total of 39 alleles was recorded (Table 1).

Diversity indexes were calculated for all VNTRs. CVN014 was the most variable locus in the analysis ($D_N = 0.9$), followed by CVN016 ($D_N = 0.74$) (Table 1).