FPD-2010-0814-ver9-Franci_1P
Type: research-article

FOODBORNE PATHOGENS AND DISEASE
Volume 00, Number 00, 2011

DOI: 10.1089/fpd.2010.0814

Original Article

Genetic Characterization of Non-O157 Verocytotoxigenic *Escherichia coli* Isolated from Raw Beef Products Using Multiple-Locus Variable-Number Tandem Repeat Analysis

Tomás Franci,[1] Andrea Mariel Sanso,[1,2] Ana V. Bustamante,[1,2] Paula M.A. Lucchesi,[1,2] and Alberto E. Parma[1]

Abstract

Verocytotoxigenic *Escherichia coli* (VTEC) can produce serious human illness linked to the consumption of contaminated food, mainly of bovine origin. There is growing concern about non-O157 VTEC serotypes, which in some countries cause severe infections in a proportion similar to O157:H7 strains. As several epidemiological studies indicated the important role of meat as the major vehicle in the transmission of this pathogen to human consumers, our aim was to investigate the genetic diversity among non-O157:H7 VTEC isolated from raw beef products. We performed a multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA), and to our knowledge, this is the first time that VTEC serotypes O8:H19, O112:H2, O113:NM, O171:NM, ONT:H7, ONT:H19, and ONT:H21 were typed by this method. MLVA typing grouped the total number of strains from this study (51) into 21 distinct genotypes, and 11 of them were unique. Several MLVA profiles were found in different serotypes, O178:H19 being the most variable. The isolates could be principally discriminated by alleles of three of seven loci studied (CVN001, CVN004, and CVN014), and on the other hand, CVN003 rendered null alleles in all the isolates. As some VNTR markers might be serotype specific, it is possible that the implementation of new VNTR loci will increase intraserotype discrimination.

Introduction

Verocytotoxigenic *Escherichia coli* (VTEC) is a zoonotic pathogen harbored by many reservoirs and transmissible to humans largely through contaminated foods, mainly of bovine origin (Karmali, 1989; Paton and Paton, 1998), although a number of infections due to nonmeat products have also been reported (Cooley *et al.*, 2007). VTEC infections can produce serious, sometimes fatal, diseases, such as hemolytic uremic syndrome (HUS). *E. coli* O157:H7 is the serotype most frequently associated with both outbreaks and sporadic cases. However, more than 100 *E. coli* serotypes have been associated with human illness (Johnson *et al.*, 2006), and therefore, there is growing concern about non-O157 VTEC serotypes, which in some countries cause severe infections in a proportion similar to O157:H7 strains (Johnson *et al.*, 2006; Coombes *et al.*, 2008).

Rhoades *et al.* (2009) reviewed information published about VTEC prevalence in cattle and beef, from the farm to the final ready-to-eat product, and noted that prevalence in the beef chain varies considerably between surveys. Several studies have confirmed that cattle also are a reservoir of VTEC in Argentina (Sanz *et al.*, 1998; Parma *et al.*, 2000; Mercado *et al.*, 2004), with prevalence values as high as 63% (Padola *et al.*, 2004). Among beef products, there are reports in which VTEC contaminate 8.4%–29% of hamburger samples, 25%–43% of ground beef samples, and 25% of retail beef cuts (Parma *et al.*, 2000; Sanz *et al.*, 2007; Etcheverría *et al.*, 2010).

Molecular typing methods are used for epidemiological investigation to trace bacterial contamination, determine the distribution of pathogens isolated from ill people, and also rule out nonrelated isolates from a particular outbreak (Foley *et al.*, 2009). Multiple-locus variable-number tandem repeats (VNTR) analysis (MLVA) has been found to be very useful in discriminating otherwise indistinguishable types in highly clonal organisms such as *E. coli* and it was first described in *E. coli* for O157 strains (Lindstedt *et al.*, 2003; Noller *et al.*, 2003; Keys *et al.*, 2005). As these protocols are specific for O157, Lindstedt *et al.* (2007) developed an MLVA method generic for *E. coli*. More recently, Miko *et al.* (2010) obtained genetic profiles of *E. coli* O26 strains by this assay, and Bustamante *et al.* (2010) compared VTEC

[1]Lab. Inmunoquímica y Biotecnología, Dto. de Sanidad Animal y Medicina Preventiva, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Pcia. de Buenos Aires, Tandil, Argentina.
[2]CONICET.

◀AU1
◀AU2

Table 1. Verocytotoxigenic *Escherichia coli* Isolates Used in This Study

Serotype	*Number of isolates*	*Source*	*Virulence profile*[a]
O8:H19	7	MM	*vtx2*, *ehxA*
	1	MM	*vtx1*, *vtx2*, *ehxA*
O22:H8	1	MM	*vtx2*
O112:H2	1	MM	*vtx2*
O113:H21	1	MM	*vtx2*, *ehxA*, *saa*
O113:NM	10	MM	*vtx2*
O171:NT	1	MM	*vtx2*
O171:NM	2	MM	*vtx2*
O174:H21	5	MM	*vtx2*
O178:H19	3	MM	*vtx2*
	2	MM	*vtx2*, *ehxA*, *saa*
ONT:H7	5	MM	*vtx2*
	1	H	*vtx2*
	1	MM	*vtx2*, *ehxA*, *saa*
ONT:H19	4	MM	*vtx2*, *ehxA*, *saa*
	1	MM	*vtx1*, *vtx2*, *ehxA*, *saa*
ONT:H21	2	MM	*vtx2*
ONT:NT	1	MM	*vtx2*
	1	MM	*vtx2*, *ehxA*
	1	H	*vtx2*, *ehxA*, *saa*

[a]Examined in previous studies.
NM, nonmotile; NT, nontypeable; MM, minced meat; H, hamburger.

isolates belonging to several serotypes, mostly obtained from bovines.

As several studies indicated the important role of meat as the major vehicle in the transmission of this pathogen to human consumers, our aim was to investigate the genetic diversity among all non-O157:H7 VTEC isolated from raw beef products in a study performed during summer 2003. As several of these isolates belonged to serotypes that have been reported from diarrhea and HUS cases worldwide (www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm) and other isolates belonged to serotypes that have not been previously typed by MLVA, our interest was to include all of them in the analysis.

Materials and Methods

Bacterial strains

Fifty-one non-O157:H7 VTEC isolates from the collection of the Laboratorio de Inmunoquímica y Biotecnología (UNCPBA, Tandil, Argentina) were investigated. They had been obtained in a previous study by Sanz *et al.* (2007) from minced meat and hamburgers. The isolates belonged to 13 serotypes and were previously analyzed by PCR for the presence of genes encoding for verocytotoxin 1 and 2 (*vtx1* and *vtx2*), intimin (*eae*), enterohemolysin (*ehxA*), and STEC autoagglutinating adhesin (*saa*) (Sanz *et al.*, 2007) (Table 1). ◀T1

MLVA typing

The seven VNTR loci studied and the primers used were those proposed by Lindstedt *et al.* (2007) and the assay was performed as described by Bustamante *et al.* (2010).

To confirm the results of the fragment analysis, representative alleles were sequenced with an ABI PRISM 3730XL genetic analyzer (Macrogen).

Data analysis

The alleles were named according to the number of tandem repeat sequences. If no amplification product was detected, the allele was designated with an arbitrary number (30). Alleles that presented partial repeats were rounded up or down to the closest complete repeat number, in agreement with Hyytiä-Trees *et al.* (2006). The diversity index (D_N), based on Nei's marker diversity, was calculated for each locus using the formula $D_N = 1 - \Sigma(fr_a)^2$, where fr_a is the allelic frequency (Noller *et al.*, 2003). Null alleles were also taken into account to determine this index.

MLVA profiles were defined with an order of the allele string that was always CVN001-CVN002-CVN003-CVN004-CVN007-CVN014-CVN015. The dendrogram was constructed using the UPGMA clustering method implemented by START

Table 2. Distribution of Alleles by Serotype and Locus, and Nei's Diversity Index by Locus

	Loci							
Serotypes	*CVN001*	*CVN002*	*CVN003*	*CVN004*	*CVN007*	*CVN014*	*CVN015*	*Total profiles*
O8:H19 ($n=8$)	7, 9	1	NA	12	6	9, 10, 11, 12	5	4
O22:H8 ($n=1$)	7	1	NA	12	6	7	5	1
O112:H2 ($n=1$)	7	1	NA	12	6	8	5	1
O113:H21 ($n=1$)	7	1	NA	9	NA	6	NA	1
O113:NM ($n=10$)	7	1	NA	12	NA, 6	8	NA, 5	2
O171:NT ($n=1$)	8	1	NA	12	6	7	5	1
O171:NM ($n=2$)	1	1	NA	12	6	7	5	1
O174:H21 ($n=5$)	9	1	NA	12	6	14, 18	5	2
O178:H19 ($n=5$)	7, 9	1	NA	11, 12	6	7, 14, 15	5	3
ONT:H7 ($n=7$)	7, 9	1	NA	12	6	5, 9, 10	5	4
ONT:H19 ($n=5$)	9	1	NA	12	6	5	5	1
ONT:H21 ($n=2$)	7	NA	NA	12	6	8, 11	5	2
ONT:NT ($n=3$)	7, 9	1	NA	12	6	5, 7, 10	5	3
Nei's diversity index	0.55	0.07	0	0.15	0.07	0.85	0.07	

NA, nonamplified product.

Vs. 1.0.5 software (Joley *et al.*, 2001). The discriminatory power of the typing method was evaluated using the Simpson's index of diversity (D_S) (Hunter and Gaston, 1988). As the dependency between isolates originating from a single sample was unknown, two different discriminatory indexes were calculated, one including all VTEC isolates and another including only a representative isolate from each MLVA profile and serotype.

Results

The 51 VTEC isolates tested could be typed by this MLVA assay and a total of 21 alleles were observed. The number of alleles detected per locus ranged between 0 (CVN003) and 11 (CVN014) (Table 2). All the samples presented a null allele at locus CVN003 (Table 2) and this suggests either locus absence or sequence polymorphism at one or both of the priming sites.

MLVA typing divided the total number of strains into 21 distinct genotypes, and 11 of them were unique to a single strain (Fig. 1). The serotypes that presented the highest diversity according to MLVA were O8:H19 and O178:H19. The former presented four of eight isolates with an identical profile, and three profiles were detected among the remaining four isolates. Among the five O178:H19 isolates, three profiles were seen (two of them shared each by two isolates and the other unique). The intraserotype differences among profiles were explained by three loci at most.

Profiles corresponding to different serotypes differed in one to three loci. Exceptionally, some profiles were shared by

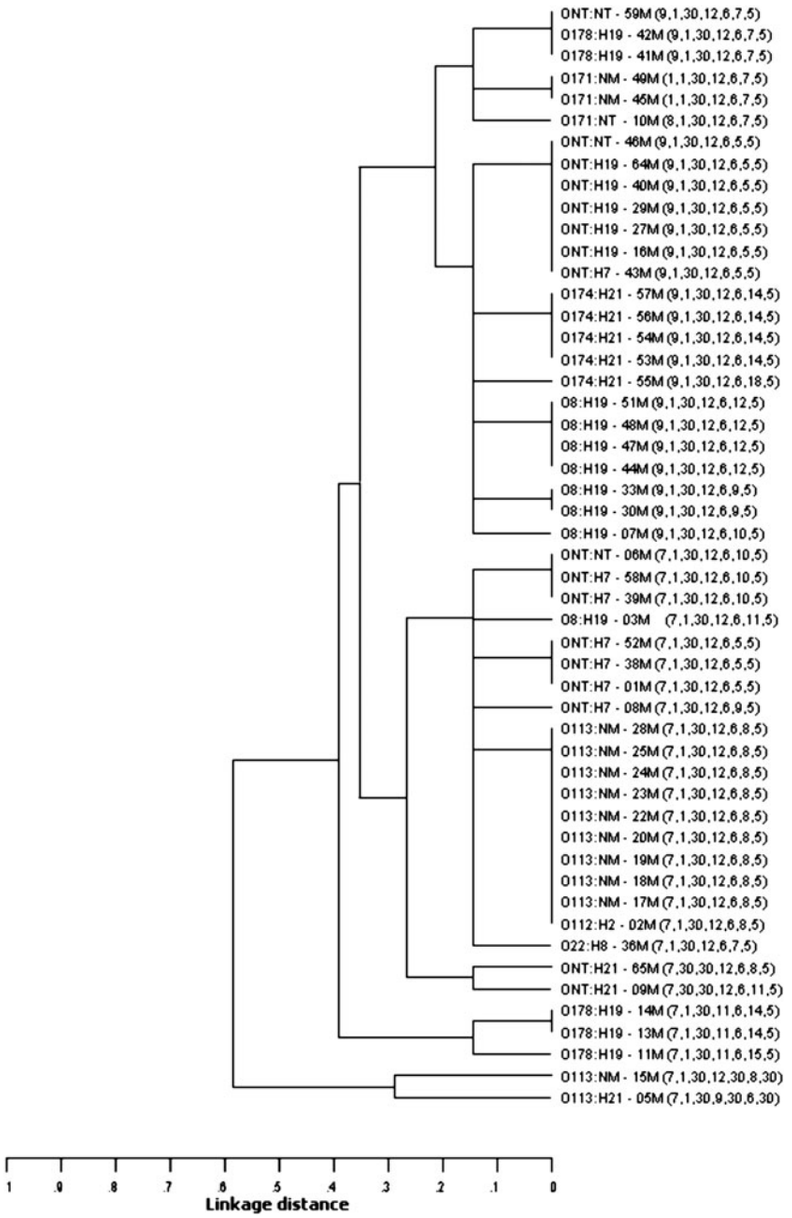

FIG. 1. Multiple-locus variable-number tandem repeat analysis–based clustering of verocytotoxigenic *Escherichia coli* non-O157:H7 isolates investigated in this study. The multiple-locus variable-number tandem repeat analysis profile comprises the allele numbers at all seven loci in the order CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, and CVN015.

isolates belonging to different serotypes. The most frequent MLVA profile was 7-1-30-12-6-8-5, shown in 10 isolates (19.6%), nine of these isolates belonged to O113:NM and one to O112:H2. The other frequent profile (9-1-30-12-6-5-5) was found in seven isolates (13.7%) (Fig. 1).

Almost all the analyzed isolates carried vtx_2, but one belonging to serotype O8:H19 and one ONT:H19 also possessed vtx_1 (Table 1). The vtx_1/vtx_2-positive isolate belonging to O8:H19 presented an MLVA profile different from the other isolates of the same serotype. Nevertheless, the profile from the remaining vtx_1/vtx_2-positive isolate was shared by several isolates.

Nei's diversity indexes calculated for all the VNTRs that could be amplified showed that CVN002 and CVN007 had the lowest values ($D_N = 0.07$), whereas CVN014 ($D_N = 0.85$) was the most polymorphic locus (Table 2). When we analyzed intraserotype loci diversity, we found that indexes notably differed among serotypes (data not shown). The isolates could be principally discriminated by alleles of loci CVN014 and CVN001, followed by those of CVN004. The most variable serotype (O178:H19) showed different alleles for these three loci, whereas O174:H21 and ONT:H21 isolates presented variability in only one locus, CVN014 (Table 2).

Simpson's index of diversity, calculated for the combined typing set, showed a value of $D_S = 0.93$ including all VTEC strains, and one of $D_S = 0.96$ when excluding replicates possibly originated from a single food sample.

Discussion

Our study compared VTEC isolates from meat products belonging to several serotypes, using MLVA, to study the genetic diversity among them. The authors who developed this assay, Lindstedt *et al.* (2007), applied it for detecting an HUS outbreak caused by *E. coli* O103:H25 in Norway and to ascertain the source of infection, cured mutton sausages (Schimmer *et al.*, 2008). To our knowledge, this is the first time VTEC serotypes O8:H19, O112:H2, O113:NM, O171:NM, ONT:H7, ONT:H19, and ONT:H21 have been typed by MLVA.

Not all the loci studied by us had an equal diversity, and moreover, they had different diversity indexes in different serotypes. In the present study, CVN014, or the alternative name O157–11 according to Keys *et al.* (2005), was the most variable locus, coinciding with the results of Lindstedt *et al.* (2007) and Bustamante *et al.* (2010). The finding of low diversity in some loci (CVN002, CVN004, CVN007, and CVN015) in relation to the values published by Lindstedt *et al.* (2007) and Bustamante *et al.* (2010) might be due to the limited number of isolates. The locus CVN003 presented a null allele for all the samples studied, coinciding with the results reported by Lindstedt *et al.* (2007) for isolates belonging to the O103 serogroup; however, amplification of this VNTR locus has been reported in other serotypes such as O145:H- and O157:H7 (Lindstedt *et al.*, 2007; Bustamante *et al.*, 2010).

According to the epidemiological information that was available, we cannot determine whether most of the isolates that formed clusters with identical MLVA profiles were derived from a unique source and constitute a single clone.

The discriminatory power evaluated by two Simpson's indexes of diversity, as described by Stakenborg *et al.* (2006), resulted in values that can be considered discriminatory ($D_S = 0.93$ and 0.96). This implies that if in the sampling some isolates represent an identical clone, the true value of this index should fall between these two estimated values.

Major advantages of MLVA are the speed of analysis, low cost, and the ability to produce numerical data that are easily portable between laboratories. Keeping in mind that the MLVA typing scheme used in this study was developed for *E. coli* and as some VNTR markers might be serotype specific, it is possible that the proposed VNTR loci are not variable enough for typing all non-O157:H7 strains. New VNTR loci for specific serotypes were recently implemented for Izumiya *et al.* (2010) and other ones are under development in our laboratory.

In conclusion, our findings corroborate the usefulness of the MLVA method as a tool for non-O157 VTEC typing and also point out that the implementation of new VNTR is needed for a more efficient MLVA typing scheme, with increased intraserotype discrimination.

Acknowledgments

The authors thank Marcelo Sanz and Alejandra Krüger for providing information about the isolates studied. This work was supported by grants from the CONICET, CIC, FONCYT, and SECAT-UNICEN.

Disclosure Statement

No competing financial interests exist.

References

Bustamante AV, Sanso AM, Lucchesi PMA, and Parma AE. Genetic diversity of O157:H7 and non O157 verocytotoxigenic *Escherichia coli* from Argentina by multiple-loci VNTR analysis (MLVA). Int J Med Microbiol 2010;300:212–217.

Cooley M, Carychao D, Crawford-Miksza L, Jay MT, Myers C, Rose C, Keys C, Farrar J, and Mandrell RE. Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. PLoS One 2007;2:e1159.

Coombes BK, Wickham ME, Mascarenhas M, Gruenheid S, Finlay BB, and Karmali MA. Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing *Escherichia coli* strains. Appl Environ Microbiol 2008;74:2153–2160.

Etcheverría AI, Padola NL, Sanz ME, Polifroni R, Krüger A, Passucci J, Rodríguez EM, Taraborelli AL, Ballerio M, and Parma AE. Occurrence of Shiga toxin-producing *E. coli* (STEC) on carcasses and retail beef cuts in the marketing chain of beef in Argentina. Meat Sci 2010;86:418–421.

Foley SL, Lynne AM, and Nayak R. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect Genet Evol 2009;9:430–440.

Hunter PR and Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988;26:2465–2466.

Hyytiä-Trees E, Smole SC, Field PA, Swaminathan B, and Ribot EM. Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis of shiga toxin-producing *Escherichia coli* O157 (STEC). Foodborne Pathog Dis 2006;3:118–131.

Izumiya H, Pei Y, Terajima J, Ohnishi M, Hayashi T, Iyoda S, and Watanabe H. New system for multilocus variable-number

tandem-repeat analysis of the enterohemorrhagic *Escherichia coli* strains belonging to three major serogroups: O157, O26, and O111. Microbiol Immunobiol 2010;54:569–577.

Johnson KE, Thorpe CM, and Sears CL. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clin Infect Dis 2006;43:1587–1595.

Joley KA, Feil EJ, Chan MS, and Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics 2001;17:1230–1231.

Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. Clin Microbiol Rev 1989;2:15–38.

Keys C, Kemper S, and Keim P. Highly diverse variable number tandem repeat loci in the *E. coli* O157:H7 and O55:7 genomes for high-resolution molecular typing. J Appl Microbiol 2005;98:928–940.

Lindstedt BA, Heir E, Gjernes E, Vardund T, and Kapperud G. DNA fingerprinting of Shiga-toxin producing *Escherichia coli* O157 based on multiple-locus variable-number tandem-repeats analysis (MLVA). Ann Clin Microbiol Antimicrob 2003;2:12.

Lindstedt BA, Thorstensen Brandal L, Aas L, Vardund T, and Kapperud G. Study of polymorphic variable-number of tandem repeats loci in the ECOR collection and in a set of pathogenic *Escherichia coli* and *Shigella* isolates for use in a genotyping assay. J Microbiol Methods 2007;69:197–205.

Mercado EC, Gioffré A, Rodríguez SM, Cataldi A, Irino K, Elizondo AM, Cipolla AL, Romano MI, Malena R, and Méndez MA. Non-O157 Shiga toxin-producing *Escherichia coli* isolated from diarrhoeic calves in Argentina. J Vet Med 2004;Series B 51:82–88.

Miko A, Lindstedt BA, Brandal LT, Løbersli I, and Beutin L. Evaluation of multiple-locus variable number of tandem-repeats analysis (MLVA) as a method for identification of clonal groups among enteropathogenic, enterohaemorrhagic and avirulent *Escherichia coli* O26 strains. FEMS Microbiol Lett 2010;303:137–146.

Noller AC, McEllistrem MC, Pacheco AG, Boxrud DJ, and Harrison LH. Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. J Clin Microbiol 2003;41:5389–5397.

Padola NL, Sanz ME, Blanco JE, Blanco M, Blanco J, Etcheverría AI, Arroyo GH, Usera MA, and Parma AE. Serotypes and virulence genes of bovine Shigatoxigenic *Escherichia coli* (STEC) isolated from a feedlot in Argentina. Vet Microbiol 2004;100:3–9.

Parma AE, Sanz ME, Blanco JE, Blanco J, Viñas MR, Blanco M, Padola NL, and Etcheverría AI. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Importance in public health. Eur J Epidemiol 2000;16:57–62.

Paton JC and Paton AW. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 1998;11:450–479.

Rhoades JR, Duffy G, and Koutsoumanis K. Prevalence and concentration of verocytotoxigenic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: a review. Food Microbiol 2009;26:357–376.

Sanz ME, Villalobo C, Elichiribehety E, and Arroyo GH. Prevalencia de *Escherichia coli* verocitotoxigénico en productos cárnicos de la ciudad de Tandil. La Ind Cárnica Lat 2007;146: 56–58.

Sanz ME, Viñas MR, and Parma AE. Prevalence of bovine verotoxin-producing *Escherichia coli* in Argentina. Eur J Epidemiol 1998;14:399–403.

Schimmer B, Nygard K, Eriksen HM, Lassen J, Lindstedt BA, Brandal LT, Kapperud G, and Aavitsland P. Outbreak of haemolytic uraemic syndrome in Norway caused by stx2-positive *Escherichia coli* O103:H25 traced to cured mutton sausages. BMC Infect Dis 2008;8:41.

Stakenborg T, Vicca J, Maes D, Peeters J, de Kruif A, Haesebrouck F, and Butaye P. Comparison of molecular techniques for the typing of *Mycoplasma hyopneumoniae* isolates. J Microbiol Methods 2006;66:263–275.

Address correspondence to: ◀AU1
Andrea Mariel Sanso ◀AU3
Lab. Inmunoquímica y Biotecnología
Dto. de Sanidad Animal y Medicina Preventiva
Facultad de Ciencias Veterinarias
Universidad Nacional del Centro de la Pcia. de Buenos Aires
Paraje Arroyo seco s/n
7000 Tandil
Argentina

E-mail: msanso@vet.unicen.edu.ar

AUTHOR QUERY FOR FPD-2010-0814-VER9-FRANCI_1P

AU1: In affiliation 1 and address of correspondence, please expand Lab., Dto., and Pcia.
AU2: Please mention the location in affiliation 2.
AU3: Please mention the degrees of the corresponding author.