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Multiplex PCR Assay for the Detection of Five Putative Virulence Genes Encoded in Verotoxigenic *Escherichia coli* Plasmids

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Abstract The aim was to perform a pentavalent PCR assay for the detection of putative virulence genes encoded in VTEC plasmids, *katP*, *espP*, *subA*, *stcE*, and *ehxA*. The five-specific primer pairs used in the assay do not interfere with each other and generate amplification products of 914, 774, 556, 399, and 262 bp. It was selected at random 39 strains belonged to 20 serotypes in order to evaluate the multiplex in a wide variety of strains. The results of this study indicate that it is possible to perform simultaneous amplification and search for recognized plasmid-encoded virulence markers from different *E. coli* serotypes and apply this technique to the genetic characterization of *E. coli* strains isolated from reservoirs, foods or patients. This complementary technique is a useful tool to detect interstrain differences for epidemiological studies and to provide information that could be related to the risk of human infection.

Introduction

Verotoxigenic *Escherichia coli* (VTEC or its synonym STEC) is a zoonotic emergent pathogen that represents an important cause of diseases in humans like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [19]. The VTEC family is very diverse, being *E. coli* O157:H7 the most common serotype associated with sporadic cases and large outbreaks of diseases in many countries [1, 44]. However, there is growing concern about the risk to human health associated with non-O157 VTEC serotypes. About 250 non-O157 VTEC serotypes have been reported and more than 100 of them have been associated with human illness [http://www.microbionet.com.au/frames/feature/vtec/brief01.html].

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The Verotoxin (Vtx) and different variants of this bacteriophage-encoded toxin are considered the cardinal virulence trait of VTEC [17]. However, additional virulence determinants also contribute to the pathogenesis of VTEC infections [3, 18]. The genes responsible for an intimate adherence of the bacteria and the attaching and effacing lesions are located on a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) [23]. The majority of VTEC serogroups related to severe disease, including the most prevalent O157:H7, possess this locus. However, some VTEC without LEE are associated with sporadic and outbreak cases of HUS [28, 39].

In addition to Vtxs, LEE, and other pathogenicity islands as well as non-LEE encoded Type III efectors, which are chromosomally encoded, all clinical isolates of *E. coli* O157:H7 possess a putative virulence plasmid called pO157. Similar plasmids are commonly present in non-O157 VTEC strains [15, 27] and a considerable variability in them has also been observed among them. Furthermore, some O26 and O145 strains that belonged to identical pulsed-field gel electrophoresis types could be further discriminated by the detection of plasmid-encoded genes [43, 47].

Several plasmid genes have been previously characterized including those that code for a haemolysin (*ehxA*) [42], a catalase-peroxidase (*katP*) [5], an extracellular serine protease (*espP*) [6], a zinc metalloprotease (*stcE*, also called *tagA*) [20, 34], a subtilase cytotoxin (*sub*AB) [36], among others. EhxA was the first described virulence factor of pO157 [42] and a sequence of the gene has been used as a diagnostic probe for *E. coli* O157:H7 and often EHEC. KatP may help *E. coli* O157:H7 colonize host intestines by

reducing oxidative stress and using the by-product oxygen in deprived oxygen conditions of the host intestine [5, 21]. EspP is known to cleave pepsinA and human coagulation factor V which could contribute to mucosal hemorrhage observed in HC [6] and influences the intestinal colonization and adherence in bovines [8]. StcE contributes to intimate adherence of this bacterium to host cells [14]. SubAB has been described in certain highly virulent VTEC strains which are negative for the LEE and shown to be cytotoxic to Vero cells and lethal for mice [26, 38].

A positive PCR reaction with primers specific for v*tx*1 or v*tx*2 can evidence the presence of VTEC in a sample, but the use of primers capable of detecting accessory virulence genes provides additional information that may also have great epidemiological value [35]. The aim of this study was to perform a multiplex PCR assay for the simultaneous detection of five putative virulence plasmid genes. This complementary technique would allow a further virulence characterization of VTEC useful to detect interstrain differences for epidemiological studies and to provide information that could be related to the risk of human infection.

Materials and Methods

Bacterial Isolates

A total of 39 VTEC strains from the collection of the Laboratorio de Inmunoquímica y Biotecnología (UNCPBA, Tandil, Argentina) were investigated. The strains belong to 20 different serotypes and were selected at random from a variety of serotypes in order to evaluate the multiplex assay. The serotypes analysed were: O2:H5, O8:H16, O8:H19, O20:H19, O22:H8, O39:H49, O79:H19, O91:H21, O112:H2, O113:H21, O113:H-, O117:H7, O141:H7, O145:H-, O157:H7, O171:H2, O171:NT (O171:H-non-typeable), O174:H21, O178:H19, ONT:H21 (O-non-typeable:H21). The strains had been isolated from cattle, patients with diarrhoea and contaminated food and previously analysed by PCR to detect the presence of genes coding for verotoxin 1 and 2 (*vtx1* and *vtx2*), intimin (*eae*), enterohaemolysin (*ehxA*), STEC autoagglutinating adhesin (*saa*) and subtilase cytotoxin (*subAB*) [4, 13, 22, 31, 32, 41]. Reference strain EDL933 (serotype O157:H7) was kindly provided by Dr. Jorge Blanco (*E. coli* Reference Laboratory, University of Santiago de Compostela, Lugo, Spain).

Multiplex PCR Design

The plasmid genes detected in this study were *kat*P (periplasmic catalase-peroxidase) [5], *subAB* (subtilase cytotoxin) [36], *esp*P (extracellular serine protease) [6], *stc*E (metalloprotease) [20], *ehx*A (enterohaemolysin A) [42]. PCR primers specific for four of these genes, were designed with reference to published sequence data for the pO157 (AF074613) [7] using Primer-BLAST software [40], from the BLAST page on the NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to get a convenient electrophoretic separation, each primer set was designed to obtain a certain amplicon size. Previously published PCR primers were used for the detection of *subA* [36]. Details of the primers nucleotide sequence, the specific gene region amplified, and the size of the PCR products are listed in Table 1.

PCR Reactions

Crude DNA extracts were obtained from a loop of frozen bacteria suspended in 500 µl of sterile water and boiled for 15 min. Each PCR reaction was carried out in a 25 µl volume containing, 1× PCR Buffer (50 mM KCl, 10 mM Tris pH9, 0.1% Triton X-100), 2 mM $MgCl_2$, 200 µM of each dNTP, 25 pmol of each primer for *subA*, *espP*, *katP*, *hlyA* genes, 5 pmol of each primer for *stcE* gene, 1U *Taq*

Table 1 Characteristics of the PCR primers used in the study

Primer name	Primer sequence (5′→3′)	Target gene	Location within gene (pb)	Predicted amplicon size (bp)	Reference
katP Fw	GCGCCAGTGGTGGTCAGCAA	*katP*	476–495	914	This study
katP Rv	ATATCGGGCTGCCGGTCCCA		1370–1389		This study
espPFw	GCTGGCAACCAGCAACAGCG	*espP*	2700–2719	774	This study
espP Rv	CGGTAGCCCGCTTCTGCACC		3452–3473		This study
subHCDF	TATGGCTTCCCTCATTGCC	*subA*	276–294	556	Paton and Paton 2005
subSCDR	TATAGCTGTTGCTTCTGACG		812–831		Paton and Paton 2005
stcE Fw	GGCTCCGGAGGTGGGGGAAT	*stcE*	1174–1193	399	This study
stcE Rv	GAAGCCGGTGGAGGAACGGC		1553–1572		This study
ehxA Fw	ACAGCTGCAAGTGCGGGTCTG	*ehxA*	865–885	262	This study
ehxA Rv	GGGATGCACTGGAGGCTGCAC		1106–1126		This study

DNA polymerase (InBio, Highway) and 2.5 μl of crude DNA extract. After an initial denaturation step of 5 min at 94°C the samples were subjected to 30 cycles, each consisting of 30 s at 94°C (denaturing), 60 s at 58°C (annealing) and 150 s at 72°C (extension). The reaction was completed with a final extension step of 5 min at 72°C. PCR products were visualized in 2% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA).2

Sensitivity of the Multiplex PCR

To assess the sensitivity of the multiplex PCR, serial tenfold dilutions of a colony of O157:H7 VTEC strain (FC O157) resuspended in 1 ml of saline solution were performed. Crude extracts of these samples were then subjected to the pentavalent PCR assay.

Results

The reaction conditions for the multiplex PCR assay were optimized to ensure that all target gene sequences could be satisfactorily amplified, rendering amplicons easily visible on a gel. First, to standardize the conditions of the assay each one of the five genes (*katP, espP, subA, stcE, ehxA*) was amplified alone, testing different annealing temperatures and primer concentrations.

The five primer pairs used in the assay did not interfere with each other and generated amplification products of 914, 774, 556, 399, and 262 bp. The genes were detected with good efficiency in the multiplex PCR and the reaction conditions resulted in strong reproducible PCR amplicons of the predicted size in all the studied isolates. The five PCR products could still be seen in the sample that contained a 10^3-fold-diluted VTEC colony (equivalent to ~10 VTEC CFU per PCR reaction), meanwhile two of them (*stcE* and *ehxA*) could be detected even at higher dilutions.

The PCR products had sizes different enough to be easily identified by agarose gel electrophoresis. In order to resolve all the bands it was essential to run 2% agarose gels at 100 V for at least 60 min (Fig. 1). Clear PCR products were obtained for all five genes from pooled DNA of one O157:H7 strain (*katP* + *espP* + *stcE* + *ehxA*+) and one O20:H19 strain (*espP* + *subA* + *ehxA*+), indicating a lack of interference between any of the primer pairs or amplicons (figure not shown).

The most commonly detected gene was *espP,* which was present in 37/39 (95%) of the VTEC strains tested. The *ehxA* gene was present in 23/39 (59%), meanwhile *subA* in 12/39 (31%), *katP* in 4/39 (10%), and *stcE* in 2/39 (Table 2). Those strains that were positive for *subA* harbored neither *stcE* nor *katP*.

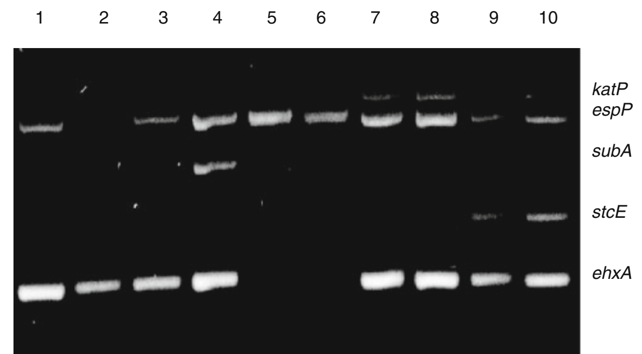

Fig. 1 Multiplex PCR of selected analysed strains. Bands corresponding to *katP, espP, subA, stcE,* and *ehxA* are indicated. *Lanes* correspond to PCR products obtained from different strains

Table 2 Putative plasmid virulence genes detected in 39 VTEC strains by the multiplex PCR analysis

Serotype	No. of strains	Genes detected by multiplex PCR				
		katP	*espP*	*subA*	*stcE*	*ehxA*
O2:H5	1	−	+	+	−	+
O8:H16	3	−	+	−	−	−
O8:H19	2	−	−	−	−	+
O20:H19	3	−	+	+	−	+
O22:H8	2	−	+	−	−	+
O39:H49	2	−	+	+	−	+
O79:H19	2	−	+	+	−	+
O91:H21	2	−	+	−	−	+
O112:H2	1	−	+	−	−	−
O113:H21	3	−	+	+	−	+
O113:H-	2	−	+	−	−	−
O117:H7	2	−	+	−	−	−
O141:H7	1	−	+	+	−	+
O145:H-	2	+	+	−	−	+
O157:H7	2	+	+	−	+	+
O171:H2	2	−	+	−	−	−
O171:NT	1	−	+	−	−	−
O174:H21	2	−	+	−	−	±[a]
O178:H19	2	−	+	−	−	−
ONT:H21	2	−	+	−	−	−
Total	39	4	37	12	2	23

[a] ± denotes the presence of the *ehxA* gene in one strain and the absence in the other one

The multiplex PCR assay allowed the detection of six plasmid profiles (Table 3). Among the strains studied, the most frequent profile was *espP* + (16/39 strains), followed by the profile *espP* + *subA* + *ehxA* + (12/39 strains).

All the strains tested had been previously screened for the presence of two of the virulence genes detected by this PCR assay (*ehxA* and *subA*) [4, 13, 22, 31, 32]. There was a total correlation between the previous results for those

Table 3 Different distribution of plasmid encoded genes observed by serotype and frecuencies

Profiles	Frecuencies	Serotypes
espP+	16/39	O8:H16, O112:H2, O113:H-, O117:H7, O171:H2, O171:NT, O174:H21, O178:H19, ONT:H21
espP+ subA+ ehxA+	12/39	O2:H5, O20:H19, O39:H49, O79:H19, O113:H21, O141:H7
espP+ ehxA+	5/39	O22:H8, O91:H21, O174:H21
ehxA+	2/39	O8:H19
katP+ espP+ ehxA+	2/39	O145:H-
katP+ espP+ stcE+ ehxA+	2/39	O157:H7

genes and those obtained by the multiplex PCR described in this study.

Discussion

Several multiplex PCRs have been described for the simultaneous gene amplification of virulence factors, traits and serotype identification in VTEC isolated from different sources [9–12, 24, 25, 29, 30, 33, 37, 45], however, none of them amplified plasmid genes exclusively. In this study, the authors developed a pentavalent PCR assay for the detection of genes encoded in VTEC plasmids, *katP, espP, subA, stcE*, and *ehxA*, designing specific primers for *katP*, *espP*, *stcE*, and *ehxA* genes and combining these primers with those proposed by Paton and Paton [36] for *subA* gene.

The results of this study indicate that it is possible to perform simultaneous amplification and search for recognized plasmid-encoded virulence markers from different *E. coli* serotypes and apply this technique to the genetic characterization of *E. coli* strains isolated from reservoirs, foods or patients. This pentavalent PCR showed high sensitivity and specificity.

The aim of the study was to develop and evaluate a multiplex PCR assay with strains selected at random and not to analyse the relationship between the plasmid genes detected by this assay and the strain serotypes. However, in this set of strains the authors identified that *espP* was the most prevalent gene, meanwhile *katP* was present only in serotypes O145:H- and O157:H7, and *stcE* only in O157:H7 strains.

Pathogenic VTEC may arise when a given set of virulence genes come together in an *E. coli* host [28], but the bacterial determinants necessary and sufficient for VTEC to cause severe disease remain unknown. The identification of such virulence determinants is expected to have important public-health implications [46]. In view of the increasing prevalence of non-O157 serotypes in human VTEC diseases [16], there is now a need for comprehensive data on the virulence properties of these VTEC strains, including those encoded by plasmids. This knowledge could allow the correlation of some patterns of plasmid-encoded virulence genes and disease severity caused by particular serotypes other than O157:H7.

This rapid PCR assay can be used in combination with, for example, the one described by Paton and Paton [35] for direct detection of VTEC. The multiplex PCR described here offers a complementary virulence characterization of VTEC in order to provide data to assess the potential risk for human infection of isolates from different sources.

References

1. Armstrong GL, Hollingsworth J, Morris JG (1996) Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol Rev 18:29–51
2. Bettelheim KA http://www.microbionet.com.au/frames/feature/vtec/brief01.html
3. Bielaszewka M, Karch H (2005) Consequences of enterohaemorrhagic *Escherichia coli* infection for the vascular endothelium. Thromb Haemost 94:12–18
4. Blanco M, Padola NL, Krüger A et al (2004) Virulence genes and intimin types of Shiga- toxinproducing *Escherichia coli* isolated from cattle and beef products in Argentina. Int Microbiol 7:269–276
5. Brunder W, Schmidt H, Karch H (1996) KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology 142:3305–3315
6. Brunder W, Schmidt H, Karch H (1997) EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mol Microbiol 24:767–778
7. Burland V, Shao Y, Perna NT et al (1998) The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. Nucleic Acids Res 26:4196–4204
8. Dziva F, Mahajan A, Cameron P et al (2007) EspP, a type V-secreted serine protease of enterohaemorrhagic *Escherichia coli* O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. FEMS Microbiol Lett 271:258–264
9. Fagan PK, Hornitzky MA, Bettelheim KA et al (1999) Detection of shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*), and Enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. Appl Environ Microbiol 65:868–872
10. Feng P, Monday SR (2000) Multiplex PCR for detection of trait and virulence factors in enterohemorrhagic *Escherichia coli* serotypes. Mol Cell Probes 14:333–337

11. Fratamico PM, Sackttey SK, Wiedmann M et al (2000) Detection of *Escherichia coli* O157:H7 by Multiplex PCR. J Clin Microbiol 33:2188–2191
12. Gannon VPJ, D'Souza S, Graham T et al (1997) Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of Enterohemorrhagic *Escherichia coli* strains. J Clin Microbiol 35:656–662
13. Granobles CV, Sanso AM, Krüger A et al (2010) Occurrence of subtilase cytotoxin and relation with other virulence factors in verocytotoxigenic *Escherichia coli* isolated from food and cattle in Argentina. Braz J Microbiol (in press)
14. Grys TE, Walters LL, Welch RA (2006) Characterization of the StcE protease activity of *Escherichia coli* O157:H7. J Bacteriol 188:4646–4653
15. Hales BA, Hart CA, Batt RM et al (1992) The large plasmids found in enterohemorrhagic and enteropathogenic *Escherichia coli* constitute a related series of transfer-defective Inc F-IIA replicons. Plasmid 28:183–193
16. Johnson KE, Thorpe CM, Sears CL (2006) The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clin Infect Dis 43:1587–1595
17. Kaper JB, Karmali MA (2008) The continuing evolution of a bacterial pathogen. Proc Natl Acad Sci USA 105:4535–4536
18. Karch H, Tarr PI, Bielaszewska M (2005) Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol 295:405–418
19. Karmali MA (1989) Infection by Vero cytotoxin-producing *Escherichia coli*. Clin Microbiol Rev 2:15–38
20. Lathem WW, Grys TE, Witowski SE et al (2002) StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. Mol Microbiol 45:277–288
21. Lim JY, Yoon JW, Hovde CJ (2010) A Brief Overview of *Escherichia coli* O157:H7 and Its Plasmid O157. J Microbiol Biotechnol 20:1–10
22. Lucchesi PMA, Krüger A, Parma AE (2006) Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food. Res Microbiol 157:263–266
23. McDaniel TK, Jarvis KG, Donnenberg MS et al (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci USA 92:1664–1668
24. Meng J, Zhao S, Doyle MP et al (1997) A multiplex PCR for identifying Shiga-like toxin-producing *Escherichia coli* O157:H7. Lett Appl Microbiol 24:172–176
25. Monday SR, Beisaw A, Feng PCH (2007) Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR. Mol Cell Probes 21:308–311
26. Morinaga N, Yahiro K, Matsuura G et al (2007) Two distinct cytotoxic activities of subtilase cytotoxin produced by Shiga-toxigenic *Escherichia coli*. Infect Immun 75:488–496
27. Naylor SW, Gally DL, Low C (2005) Enterohaemorrhagic *E. coli* in veterinary medicine. Int J Med Microbiol 295:419–441
28. Newton HJ, Sloan J, Bulach DM et al (2009) Shiga toxin–producing *Escherichia coli* strains negative for Locus of Enterocyte Effacement. Emerg Infect Dis 15:372–380
29. Osek J (2002) Rapid and specific identification of Shiga toxin-producing *Escherichia coli* in faeces by multiplex PCR. Lett Appl Microbiol 34:304–310
30. Osek J (2003) Development of a multiplex PCR approach for the identification of Shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. J Appl Microbiol 95: 1217–1225
31. Padola NL, Sanz ME, Blanco JE et al (2004) Serotypes and virulence genes of bovine shigatoxigenic *Escherichia coli* (STEC) isolated from a feedlot in Argentina. Vet Microbiol 100:3–9
32. Parma AE, Sanz ME, Blanco JE et al (2000) Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Importance in public health. Eur J Epidemiol 16:757–762
33. Pass MA, Odedra R, Batt RM (2000) Multiplex PCRs for identification of *Escherichia coli* virulence genes. J Clin Microbiol 38:2001–2004
34. Paton AW, Paton JC (2002) Reactivity of convalescent-phase hemolytic-uremic syndrome patient sera with the megaplasmid-encoded TagA Protein of shiga toxigenic *Escherichia coli* O157. J Clin Microbiol 40:1395–1399
35. Paton AW, Paton JC (2002) Direct detection and characterization of shiga toxigenic *Escherichia coli* by Multiplex PCR for *stx*1, *stx*2, *eae*, *ehxA*, and *saa*. J Clin Microbiol 40:271–274
36. Paton AW, Paton JC (2005) Multiplex PCR for direct detection of Shiga toxigenic *Escherichia coli* strains producing the novel subtilase cytotoxin. J Clin Microbiol 43:2944–2947
37. Paton JC, Paton AW (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 11:450–479
38. Paton AW, Srimanote P, Talbot UM et al (2004) A new family of potent AB5 cytotoxins produced by Shiga toxigenic *Escherichia coli*. J Exp Med 200:35–46
39. Paton AW, Woodrow MC, Doyle RM et al (1999) Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolyticuremic syndrome. J Clin Microbiol 37:3357–3361
40. Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, Totowa NJ (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ, pp 365–386
41. Sanz ME, Villalobo C, Elichiribehety E et al (2007) Prevalencia de *Escherichia coli* verocitotoxigénico en productos cárnicos de la ciudad de Tandil. La Ind Cárnica Lat 146:56–58
42. Schmidt H, Beutin L, Karch H (1995) Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 Strain EDL 933. Infect Immun 63:1055–1061
43. Sonntag AK, Prager R, Bielaszewska M et al (2004) Phenotypic and genotypic analyses of enterohemorrhagic *Escherichia coli* O145 strains from patients in Germany. J Clin Microbiol 42:954–962
44. Tarr PI (1995) *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin Infect Dis 20: 1–8
45. Wang G, Clark CG, Rodgers FG (2002) Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by Multiplex PCR. J Clin Microbiol 40:3613–3619
46. Wickham ME, Lupp C, Mascarenhas M et al (2006) Bacterial genetic determinants of Non-O157 STEC outbreaks and hemolytic-uremic syndrome after infection. J Infect Dis 194:819–827
47. Zhang WL, Bielaszewska M, Liesegang A et al (2000) Molecular characteristics and epidemiological significance of Shiga toxin-producing *Escherichia coli* O26. J Clin Microbiol 38:2134–2140