NOTE / NOTE

DNA sequence of the *Escherichia coli* O103 O antigen gene cluster and detection of enterohemorrhagic *E. coli* O103 by PCR amplification of the *wzx* and *wzy* genes

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Abstract: *Escherichia coli* serogroup O103 has been associated with gastrointestinal illness and hemolytic uremic syndrome. To develop PCR-based methods for detection and identification of this serogroup, the DNA sequence of the 12 033-bp region containing the O antigen gene cluster of *Escherichia coli* O103 was determined. Of the 12 open reading frames identified, the *E. coli* O103 *wzx* (O antigen flippase) and *wzy* (O antigen polymerase) genes were selected as targets for development of both conventional and real-time PCR assays specific for this serogroup. In addition, a multiplex PCR targeting the Shiga toxin (Stx) 1 (stx_1), Shiga toxin 2 (stx_2), *wzx*, and *wzy* genes was developed to differentiate Stx-producing *E. coli* O103 from non-toxigenic strains. The PCR assays can be employed to identify *E. coli* serogroup O103, replacing antigen-based serotyping, and to potentially detect the organism in food, fecal, or environmental samples.

Key words: real-time polymerase chain reaction, *E. coli* typing, *E. coli* O103, O antigen DNA sequence.

Résumé : Le sérogroupe O103 de *Escherichia coli* a été associé avec des maladies gastro-intestinales et le syndrome hémolytique urémique. Afin d'élaborer des méthodes de détection basée sur le PCR et d'identifier ce sérogroupe, la séquence d'ADN de la région de 12 033 pb contenant le groupe de gènes de l'antigène O d'*Escherichia coli* O103 a été déterminée. Des 12 cadres de lecture ouverts identifiés, les gènes *wzx* (flippase de l'antigène O) et *wzy* (polymérase de l'antigène O) de *E. coli* O103 ont été sélectionnés comme cibles pour l'élaboration des tests de PCR conventionnels et en temps réel spécifiques à ce sérogroupe. De plus, un PCR multiplex ciblant les gènes de la toxine Shiga 1 (stx_1), de la toxine Shiga 2 (stx_2) et de *wzx* et *wzy* fut conçu afin de différencier le *E. coli* O103 produisant la Stx des souches non-toxinogènes. Les tests de PCR peuvent être employés afin d'identifier le sérogroupe O103 de *E. coli*, remplaçant le sérotypage basé sur les antigènes, et de détecter potentiellement l'organisme dans les échantillons alimentaires, fécaux et environnementaux.

Mots clés : réaction de la polymérase en chaîne en temps réel, typage de *E. coli*, *E. coli* O103, séquence d'ADN de l'antigène O.

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Serotyping of *Escherichia coli* isolates is a traditional typing method involving antigen agglutination using antisera raised in rabbits against the 179 different O surface polysaccharide antigens. However, serotyping is time consuming and labor intensive and is often nonspecific, as cross reactions of antisera with multiple O serogroups frequently occurs. Furthermore, the typing antisera can only be produced by specialized laboratories that have animal facilities. Genes involved in the synthesis of the O-specific polysaccharide are located in the O antigen gene cluster between the *galF* and *gnd* genes on the *E. coli* chromosome (Samuel and Reeves 2003). Knowledge of the DNA sequence of the cluster permits identification of unique genes or sequences that can be used to design serogroup-specific PCR assays. These assays can be employed for detection, as well as typing, of *E. coli* as an alternative to serotyping. Several O antigen gene clusters have already been sequenced, including O26, O55, O91, O104, O111, O113, O121, and

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O157, and serogroup-specific PCR assays based on genes in the respective O antigen clusters have been developed (Wang et al. 1998, 2001, 2002; Wang and Reeves 1998; Paton and Paton 1999; D'Souza et al. 2002; Perelle et al. 2002, 2004; Fratamico et al. 2003).

Enterohemorrhagic *Escherichia coli* (EHEC) serogroup O103 strains, in particular Shiga toxin-producing serotypes O103:H2, O103:H-, O103:H18, O103:H21, and O103:H25 strains, have been associated with cases of bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) worldwide (Mariani-Kurkdjian et al. 1993; Luzzi et al. 1995; Tarr et al. 1996; Caprioli et al. 1997; Beutin et al. 1998; Meng et al. 1998). Additionally, a Shiga toxin 1-producing *E. coli* O103:H2 strain caused HUS in a child with a urinary tract infection with no prodromal diarrhea (Tarr et al. 1996). In Germany and Italy, for example, there has also been a notable increase in cases of disease caused by *Escherichia coli* O103 and other non-O157 EHEC strains in recent years, partly because of an increased awareness of these organisms in causing human illness, and partly because laboratories are making greater efforts to seek out these pathogens (Caprioli et al. 1997; Beutin et al. 1998). *Escherichia coli* O103 has also been isolated from healthy animals, as well as animals with diarrhea, including cattle, sheep, and rabbits (Bettelheim et al. 2001; Urdahl et al. 2002; Jenkins et al. 2003).

Over 200 Shiga toxin-producing *E. coli* (STEC) serotypes (combination of O and H antigens) have been identified, and over 100 have caused illness in humans and animals (World Health Organization 1998; Nataro and Kaper 1999). Within many serogroups identified as STEC, however, both Shiga toxin-positive and Shiga toxin-negative strains can be found; therefore, knowledge of the serogroup is not always sufficient to identify a strain as a STEC. The term EHEC refers to STEC serotypes that share the same clinical, pathogenic, and epidemiologic features with *E. coli* O157:H7, the EHEC serotype most frequently associated with human illness in the U.S. However, in South America, Europe, and Australia, as well as in the U.S., non-O157 EHEC have become an important public health problem (Beutin et al. 1998; López et al. 1998; Elliott et al. 2001; Gerber et al. 2002). Approximately 20% to 25% of the cases of HUS in the U.S., and the majority of HUS cases in many other countries, are likely caused by non-O157 EHEC (Nataro and Kaper 1999). Unlike *E. coli* O157:H7 strains, however, which generally do not ferment sorbitol or have β-glucuronidase activity, the non-O157 STEC do not have identifiable biochemical markers to facilitate screening for and identification of these pathogens. Detection of non-O157 STEC requires testing for the presence of the Shiga toxins or for the Shiga toxin genes, which is followed by serogrouping of the strain using antisera produced against the ~179 different *E. coli* serogroups. The incidence of disease caused by non-O157 EHEC is likely underestimated due to the lack of simple, rapid, and reliable methods available for detection and typing of these pathogens. Therefore, the objective of the current study was to sequence the O antigen gene cluster of an *E. coli* O103 strain and develop conventional PCR and real-time PCR assays using primers based on unique gene sequences in the cluster to facilitate detection and typing of *E. coli* O103. *Escherichia coli* O103:H8 strain H515b (obtained from World Health Organization; Ørskov et al. 1977) was used for DNA sequencing. The bacteria used to test for specificity of the PCR assays were obtained from the strain collections at the Gastroenteric Disease Center at The Pennsylvania State University (University Park, Pa.) and the Microbial Food Safety Research Unit at the Eastern Regional Research Center (Wyndmoor, Pa.).

DNA sequencing and analyses

Escherichia coli O103:H8 strain H515b was grown for 18 h in Luria–Bertani broth (Difco, Detroit, Mich.) at 37 °C, and sequencing of the O antigen gene cluster was performed as described previously (Fratamico et al. 2003). The assembled sequences were imported into Artemis (Rutherford et al. 2000), the open reading frames (ORFs) were located, and the putative coding regions were analyzed using the NCBI BLASTX program against the non-redundant (nr) database (January 15, 2004) (Altschul et al. 1997). Analysis of the DNA sequence of the 12 033-bp region containing the O antigen gene cluster of *E. coli* O103:H8 strain H515b showed that it contained 12 complete ORFs, with all having the same transcriptional direction (GenBank acc. No. AY532664). The genes within the cluster, identified with varying degrees of precision and named in accordance with the system proposed by Reeves et al. (1996), are shown in Table 1. The *E. coli* O103 O antigen gene cluster had features common to other O antigen gene clusters, which generally include nucleotide sugar pathway genes, sugar transferase genes, and O unit processing genes. The *E. coli* O103 cluster consisted of transferase genes, a gene coding for the O antigen flippase (*wzx*) that transports the repeat sugar units across the cytoplasmic membrane, and a gene coding for the O antigen polymerase (*wzy*) that polymerizes the repeat units. Analyses using HMMTOP 2.0 (Tusnády and Simon 2001; http://www.enzim.hu/hmmtop) predicted *wzx* and *wzy* to encode integral inner membrane proteins with 12 and 10 transmembrane segments, respectively. The *rmlB* (dTDP-glucose-4,6-dehydratase) and *rmlA* (D-glucose-1-phosphate thymidyltransferase) genes shared 93% and 78% identity, respectively, with the *rmlB* and *rmlA* of *E. coli* O91 (Perelle et al. 2002). The UDP-galactose 4-epimerase, the product of *galE*, catalyzes the conversion of UDP galactose to UDP glucose and the reverse reaction. Sequence analyses indicated that the *wzx* and *wzy* genes of *E. coli* O103:H8 strain H515b were specific for this strain; therefore, these sequences were selected as target genes for the *E. coli* O103-specific PCR assays. The structure of the *E. coli* O103 O-specific polysaccharide has not yet been reported.

Selection of PCR primers and specificity testing

In a number of studies, PCR assays targeting the *E. coli* *wzx* and *wzy* genes were found to be serogroup specific (Wang et al. 1998, 2001; Wang and Reeves 1998; Fratamico et al. 2003). Sequence similarity analyses were performed comparing the *E. coli* O103 *wzx* and *wzy* genes to similar genes in other *E. coli* serogroups, and results demonstrated that these genes were suitable targets for *E. coli* O103-specific PCR assays. Oligonucleotide primers, complementary to the *E. coli* O103 *wzx* and *wzy* genes, were designed and used in PCR assays to determine the specificity for this serogroup (Table 2). Template DNA from the bacteria was prepared by mixing a colony in 50 μL of sterile distilled

Table 1. Summary of genes in *Escherichia coli* O103 O antigen gene cluster.

ORF	Proposed gene name	Location	No. of amino acids	Putative function	Most significant homologs (acc. No.) % identity/% similarity
1	*rmlB*	107–1183	358	dTDP-glucose-4,6-dehydratase	RmlB, *E. coli* O91 (AAK60448), 93/94
2	*rmlA*	1180–2052	290	D-Glucose-1-phosphate thymidylyltransferase	RmlA, *E. coli* O91 (AAK60449), 78/89
3	*wbtA*	2045–2452	135	dTDP-6-deoxy-3,4-keto-hexulose isomerase	FdtA, *Aneurinibacillus thermoaerophilus* (AAO06351), 58/79
4	*wbtB*	2445–2978	177	Butyryltransferase	WbsC, *E. coli* O91 (AAK60452), 45/66
5	*wbtC*	2975–4096	373	Aminotransferase	WblQ, *Photorhabdus luminescens* (NP_931971), 67/81
6	*wzx*	4084–5349	421	O antigen flippase	WzxB, *Photorhabdus luminescens* (NP_931972), 36/58
7	*wbtD*	5353–6339	328	Glycosyl transferase	Eps9I, *Streptococcus thermophilus* (AF454499), 25/43
8	*wbtE*	6342–7232	296	Glycosyl transferase	WbsA, *Campylobacter jejuni* (AAM90644), 22/40
9	*wzy*	7232–8380	382	O antigen polymerase	Wzy, *Salmonella choleraesuis* (A47677), 25/50
10	*wbtF*	8383–9474	363	Glycosyl transferase	WbeiM, *Edwardsiella ictaluri* (AAL25631), 42/63
11	*wbtG*	9660–10781	366	Galactosyl transferase	RfpB protein, *Shigella dysenteriae* (AAC60480), 47/65
12	*galE*	10860–11888	342	UDP-galactose-4-epimerase	GalE, *Escherichia coli* (AAO37702), 66/80

Table 2. Oligonucleotide primers used for amplification of the *E. coli* O103 *wzx* and *wzy* genes and stx_1 and stx_2 genes.

Oligonucleotide primers	Sequence (5′ to 3′)	Target genes/base positions of primers (GenBank acc. No.)	Expected sizes of PCR products (bp)
O103wzxF	TTGGAGCGTTAACTGGACCT	*wzx*/4769–5089	321
O103wzxR	GCTCCCGAGCACGTATAAG		
O103wzyF	ATACAAATGGCGTGGATTGG	*wzy*/7362–7641	280
O103wzyR	GCCAGTAATTGACGTAACTGCTC		
STXI-F2[a]	CTCGACTGCAAAGACGTATG	stx_1/418–616 (AAA98100.1)	199
STXI-R2	TCGTTCAACAATAAGCCGTA		
STXII-F2	CTTTTCGACCCAACAAAGTT	stx_2/322–478 (CAA30715.1)	157
STXII-R2	ATCAAGCCCTCGTATATCCA		

[a]Primers targeting stx_1 and stx_2 were tested for specificity using a collection of laboratory clinical and food isolates of Shiga toxin-producing *E. coli* O157:H7 and non-O157:H7 and also non-*E. coli* bacteria.

water and heating at 100 °C for 20 min. The PCR at the Gastroenteric Disease Center (University Park, Pa.) was performed using a RapidCycler (Idaho Technology, Inc., Salt Lake City, Utah) using 11 µL total reaction volumes. The PCR mixture consisted of 3 µL of template DNA, 0.5 µmol/L of primers (Integrated DNA Technologies, Inc., Coralville, Iowa; Table 2), 0.18 mmol/L of each of the 4 dNTPs, 3.0 mmol/L $MgCl_2$, 0.4 U *Taq* DNA polymerase (PGC Scientifics, Frederick, Md.), 50 mmol/L Tris (pH 8.3), 250 µg/mL BSA, 2% sucrose, and 0.1 mmol/L Cresol Red (Idaho Technology, Inc.). The PCR assays were performed using primer sets for *wzx* or for *wzy* separately in each of the reactions (singleplex assays). The thermal cycling protocol was performed using the rapid cycle DNA amplification method (Wittwer et al. 1994), and consisted of an initial denaturation step at 94 °C for 30 s, followed by 30 cycles of template denaturation at 94 °C, primer annealing at 57 °C, and extension at 72 °C for 13 s. The PCR products were visualized following electrophoresis through 1% agarose gels stained with ethidium bromide.

Of 60 *E. coli* strains isolated from humans, animals, and water, and serogrouped as *E. coli* O103 by conventional serotyping at the Gastroenteric Disease Center, all tested positive by the singleplex PCR assays for the presence of the *wzx* and *wzy* genes using the primer sets designed in this study (Table 3). Results showed 100% specificity for *E. coli* O103 with no amplification of products of the expected sizes of 321 bp (*wzx*) or 280 bp (*wzy*) using DNA from non-O103 *E. coli* strains or strains of other bacterial genera (Table 3). These findings indicate that the PCR assays are suitable for detection and DNA-based typing of *E. coli* O103.

Multiplex PCR

Identification of a strain as *E. coli* serogroup O103 does not necessarily indicate that the strain is a potential human pathogen, thus the determination of the presence of serogroup-specific genes, as well as the presence of the Shiga toxin genes and (or) other virulence genes is important. Multiplex PCR assays were performed targeting both the *wzx* and *wzy* genes in a single PCR assay and targeting the Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), *wzx*, and *wzy* genes in a single assay. The PCR testing was performed using a Smart Cycler (Cepheid, Sunnyvale, Calif.) with a cycling protocol consisting of an initial denaturation step at 94 °C for 120 s followed by 35 cycles of template denaturation at 94 °C for

Table 3. Specificity testing using primers for amplification of the *E. coli* O103 *wzx* and *wzy* genes by singleplex PCR, multiplex PCR, and real-time PCR using SYBR Green I.

		Singleplex PCR primers		Multiplex PCR primers				Real-time PCR primers	
Bacterial strains	Serotype	O103wzx	O103wzy	O103wzx	O103wzy	STXI	STXII	O103wzx	O103wzy
E. coli (60 strains)[a]	O103	+	+						
E. coli[b]	O1 to O173	–	–						
E. coli	OX3	–	–						
E. coli	OX6	–	–						
E. coli	OX7	–	–						
E. coli	OX9	–	–						
E. coli	OX10	–	–						
E. coli	OX13	–	–						
E. coli	OX18	–	–						
E. coli	OX19	–	–						
E. coli	OX21	–	–						
E. coli	OX23	–	–						
E. coli	OX25	–	–						
E. coli	OX28	–	–						
E. coli	OX38	–	–						
E. coli	OX43	–	–						
Citrobacter braakii (1 strain)		–	–						
Citrobacter freundii (2 strains)		–	–						
Erwinia carotovora (1 strain)		–	–						
Klebsiella pneumoniae (1 strain)		–	–						
Listeria monocytogenes (1 strain)		–	–						
Pseudomonas aeruginosa (2 strains)		–	–						
Pseudomonas fluorescens (1 strain)		–	–						
Salmonella Enteriditis (1 strain)		–	–						
Salmonella Typhimurium (3 strains)		–	–						
Salmonella Worthington (1 strain)		–	–						
Serratia liquefaciens (1 strain)		–	–						
Shigella boydii (2 strains)		–	–						
Shigella dysenteriae (1 strain)		–	–						
Shigella flexneri (3 strains)		–	–						
Shigella sonnei (2 strains)		–	–						
Staphylococcus aureus (1 strain)		–	–						
Vibrio cholerae (1 strain)		–	–						
Yersinia enterocolitica (2 strains)		–	–						
E. coli	O26:H(-)			–	–	+	+	–	–
E. coli	O26:H2			–	–	+	–	–	–
E. coli	O26:H11			–	–	+	–	–	–
E. coli	O26:H11			–	–	+	–	–	–
E. coli	O26:H11			–	–	–	+	–	–
E. coli	O26:H11			–	–	–	+	–	–
E. coli	O26:H11			–	–	+	+	–	–
E. coli	O26:H11			–	–	+	–	–	–
E. coli	O69			–	–	+	–	–	–

Table 3 (*concluded*).

Bacterial strains	Serotype	Singleplex PCR primers		Multiplex PCR primers				Real-time PCR primers	
		O103wzx	O103wzy	O103wzx	O103wzy	STXI	STXII	O103wzx	O103wzy
E. coli	O70			–	–	–	–	–	–
E. coli	O85:NM			–	–	+	–	–	–
E. coli	O98			–	–	+	–	–	–
E. coli	O103			+	+	+	–	+	+
E. coli	O103			+	+	+	–	+	+
E. coli	O103:H2			+	+	+	–	+	+
E. coli	O103:H6			+	+	+	–	+	+
E. coli	O103:H25			+	+	+	–	+	+
E. coli	O111:H(-)			–	–	+	–	–	–
E. coli	O111:H(-)			–	–	–	+	–	–
E. coli	O118			–	–	+	–	–	–
E. coli	O119			–	–	+	–	–	–
E. coli	O119			–	–	+	–	–	–
E. coli	O121:H19			–	–	+	–	–	–
E. coli	O145:H(-)			–	–	+	+	–	–
E. coli	O165:H26			–	–	+	–	–	–
E. coli	OX13			–	–	–	–	–	–
E. coli	O negative			–	–	–	–	–	–
E. coli	O negative			–	–	–	–	–	–
E. coli	O negative			–	–	–	–	–	–
Citrobacter braakii (1 strain)				–	–	–	–	–	–
Citrobacter freundii (2 strains)				–	–	–	–	–	–
Erwinia carotovora (1 strain)				–	–	–	–	–	–
Klebsiella pneumoniae (1 strain)				–	–	–	–	–	–
Listeria monocytogenes (1 strain)				–	–	–	–	–	–
Pseudomonas aeruginosa (2 strains)				–	–	–	–	–	–
Pseudomonas fluorescens (1 strain)				–	–	–	–	–	–
Salmonella Enteriditis (1 strain)				–	–	–	–	–	–
Salmonella Typhimurium (2 strains)				–	–	–	–	–	–
Salmonella Worthington (1 strain)				–	–	–	–	–	–
Serratia liquefaciens (1 strain)				–	–	–	–	–	–
Shigella boydii (1 strains)				–	–	–	–	–	–
Shigella dysenteriae (1 strain)				–	–	–	–	–	–
Shigella flexneri (2 strains)				–	–	–	–	–	–
Shigella sonnei (2 strains)				–	–	–	–	–	–
Yersinia enterocolitica (2 strains)				–	–	–	–	–	–

Note: +, positive; –, negative; no result shown, not tested.

[a]The 60 strains of *E. coli* O103 were isolated from humans, animals, and water.

[b]Strains tested included 1 or more strains of the different *E. coli* serogroups, including field isolates and *E. coli* used as standard strains for serotyping assays. Serogroups included O1 to O173 except O103, which is listed separately, and O14, O31, O47, O67, O72, O93, O94, and O122, since these serogroup designations have been cancelled (Ørskov et al. 1977).

20 s, primer annealing at 57 °C for 60 s, and primer extension at 72 °C for 60 s, and a final extension step at 72 °C for 600 s. The PCR mixture (25 μL total reaction volume) consisted of 2.5 μL of template DNA, 0.5 μmol/L of each of the primers (Table 2; Integrated DNA Technologies, Inc., and Synthegen, Houston, Tex.), and OmniMix HS beads (Fisher Scientific, Pittsburgh, Pa.), a lyophilized predispensed blend of reagents containing 0.2 mmol/L of each of the 4 dNTPs,

4.0 mmol/L $MgCl_2$, 1.5 U hot start *Taq* DNA polymerase, 25 mmol/L HEPES (pH 8.0), rehydrated as instructed by the manufacturer. The PCR products were visualized following electrophoresis through 1.5% agarose gels stained with ethidium bromide.

The PCR products of the *wzx* and *wzy* genes were amplified at equal intensity in the multiplex PCR using *E. coli* O103:H8 strain H515b and *E. coli* O103:H3,18 strain 97.0660 DNA (Fig. 1). The PCR products of the expected sizes of 321 bp (*wzx*) and 280 bp (*wzy*) were not visible using DNA from non-O103 *E. coli* and other bacterial genera (Fig. 1 and Table 3). The PCR results targeting the stx_1 and stx_2 genes (using primers listed in Table 2) showed that *E. coli* O103:H8 strain H515b does not harbor either of these 2 genes (data not shown). Using DNA from 2 clinical isolates, *E. coli* O103:H25 strain 96-1113 (obtained from the Laboratory Centre for Disease Control, Ottawa, Ont.) and *E. coli* O103:H2 (obtained from Dr. Phillip Tarr, University of Washington) (Tarr et al. 1996), in a multiplex PCR assay targeting *wzx*, *wzy*, stx_1, and stx_2, products for *wzx*, *wzy*, and stx_1 (321-, 280-, and 199-bp products, respectively) were obtained for both strains (Fig. 2). Thus, the 2 clinical *E. coli* O103 isolates possessed stx_1 but not stx_2. All of the non-O103 strains tested by the multiplex PCR that were positive for either stx_1, stx_2, or both toxin genes were clinical isolates (Table 3). None of the non-O103 strains, however, were positive for the O103 *wzx* and *wzy* genes by the multiplex PCR.

Real-time PCR using SYBR Green I

The real-time PCR assays were performed using the Smart Cycler and 25 μL total reaction volumes. The PCR mixture consisted of 2.5 μL of template DNA, 0.5 μmol/L of primers, 0.125X SYBR Green I dye (10 000×; Molecular Probes, Inc., Eugene, Ore.), and OmniMix HS lyophilized beads. Primer sets for amplification of the *wzx* and *wzy* sequences were used separately in the PCR reactions. The cycling conditions consisted of an initial denaturation step at 94 °C for 120 s followed by 35 cycles of template denaturation at 94 °C for 20 s, primer annealing at 60 °C for 60 s, and extension at 72 °C for 60 s, and a final extension step at 72 °C for 600 s. After the last cycle was completed, melting curves of the PCR products were produced by plotting the fluorescence intensity against temperature, as the temperature was increased from 60 to 95 °C at 0.2 °C/s. The first derivative (dI/dT) of the inflection point of the melt curve (melting temperature (T_m) of the product) was calculated by the Smart Cycler software. On occasion, PCR products were also visualized following electrophoresis through 1.5% agarose gels stained with ethidium bromide to confirm the presence of products of the expected sizes. Real-time PCR using SYBR Green I and primers sets O103wzx and O103wzy showed positive results only with *E. coli* O103 strains (Table 3).

To determine the sensitivity of the real-time PCR assays, template DNA was prepared by suspending a colony from tryptic soy agar plates in 300 μL of sterile water, and 100 μL were removed to perform serial dilutions to determine the number of bacteria in each dilution. The remaining 200 μL were centrifuged at 16 000*g* for 2 min, and 200 μL of PrepMan Ultra reagent (Applied Biosystems, Foster City, Calif.) were added to the cell pellet. The DNA extraction

Fig. 1. Agarose gel electrophoresis showing results of a multiplex PCR assay using primers for the *wzx* (O103 wzxF and O103wzxR) and *wxy* genes (O103 wzyF and O103wzyR). Lane 1, *Escherichia coli* O103:H8 strain H515b; lane 2, *E. coli* O103:H3,18 strain 97.0660 (both using *wzx* primers only); lane 3, *E. coli* O103:H8 strain H515b; lane 4, *E. coli* O103:H3,18 strain 97.0660 (both using *wzy* primers only), and multiplex PCR (*wzx* and *wzy* primers) using DNA from: lane 5, *E. coli* O103:H8 strain H515b; lane 6, *E. coli* O103:H3,18 strain 97.0660; lane 7, *E. coli* O69:H42 strain 95.0790; lane 8, *Salmonella Enteriditis* strain 1952; lane 9, *Pseudomonas aeruginosa* strain 813; lane 10, *Shigella flexneri* strain 5348; lane 11, *Listeria monocytogenes* strain 524.

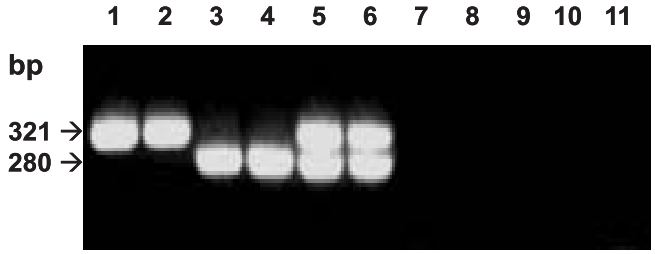
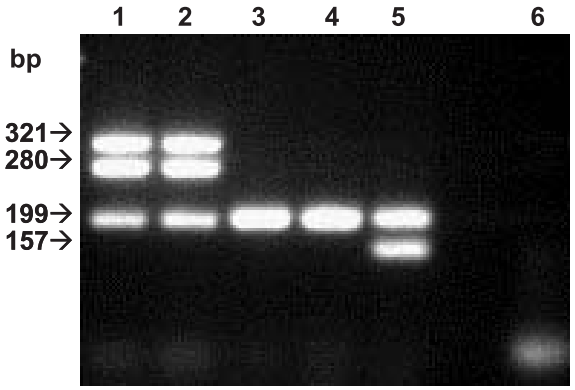


Fig. 2. Agarose gel electrophoresis showing multiplex PCR results using DNA from *E. coli* O103:H25 strain 96-1113 (lane 1) and *E. coli* O103:H2 (Tarr et al. 1996) (lane 2), isolated from patients with HC and HUS, respectively. Primers targeting the stx_1 and stx_2 genes and the *E. coli* O103:H8 strain H515b *wzx* and *wzy* genes were used (Table 2). Lane 3, DNA from *E. coli* O103:H25 strain 96-1113 using primers targeting the stx_1 and stx_2 genes; and lane 4, *E. coli* O103:H2 (Tarr et al. 1996), also using primers targeting the stx_1 and stx_2 genes. Lane 5, DNA from control strain, *E. coli* O157:H7 380-94, which harbors stx_1 and stx_2; lane 6, no template (H_2O) control.



was performed following the manufacturer's instructions. The DNA was serially diluted 10-fold with sterile water and used as the template in the PCR. The number of bacteria in each dilution was determined by plating onto tryptic soy agar plates. Cell concentrations ranging from ~1.5×10^9 to 1.5 CFU/mL were used to generate the standard curve, which was determined by plotting the cycle threshold values against $\log_{10}$ concentration of the bacteria in each dilution. The data were analyzed statistically to determine the correlation coefficients (r^2 = 0.993 and 0.981 for the standard curves for the PCR targeting *wzx* and *wzy*, respectively), which indicated the consistency of the replicates in the

reactions. A linear relationship was obtained over a range of ~10^3 to 10^9 CFU/mL, and the detection limit was ~1.5×10^3 CFU/mL for the real-time PCR assays targeting *wzx* and *wzy* (data not shown). The T_m value for the *wzx* PCR product was 83.9 ± 0.3 °C and for the *wzy* product was 80.3 ± 0.2 °C; therefore, the real-time PCR can easily distinguish the 2 products (data not shown). TaqMan probes, molecular beacons, or other real-time PCR fluorogenic techniques can also potentially be employed in real-time PCR assays targeting the *E. coli* O103 *wzx* and (or) *wzy* genes.

In conclusion, a 12 033-bp DNA region containing the *E. coli* O103 O antigen gene cluster was sequenced and shown to comprise 12 genes. Two genes, *wzx* and *wzy*, with sequences highly specific (no false negative or false positive PCR results) for *E. coli* O103 provided the basis for the development of *E. coli* O103-specific singleplex, multiplex (targeting *wzx*, *wzy*, stx_1, and stx_2), and real-time PCR assays. Use of these PCR assays enhances the ability to detect, identify, and type this serogroup, eliminating the use of the labor-intensive serotyping procedure and of assays based on the production of the Shiga toxins. In the future, the development of DNA microarrays harboring probes specific for *wzx*, *wzy*, or other genes in the O antigen gene clusters of each of the *E. coli* serogroups could greatly facilitate *E. coli* typing.

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