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The DNA Sequence of the *Escherichia coli* O22 O-Antigen Gene Cluster and Detection of Pathogenic Strains Belonging to *E. coli* Serogroups O22 and O91 by Multiplex PCR Assays Targeting Virulence Genes and Genes in the Respective O-Antigen Gene Clusters

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Abstract Multiplex polymerase chain reaction (PCR) assays were developed for detection of pathogenic strains belonging to *Escherichia coli* serogroups O22 and O91. The O-antigen gene cluster of *E. coli* O22 was sequenced to identify genes that could be employed as targets for serogroup-specific PCR assays. The *wzx* and *wzy* genes in the O-antigen gene clusters of *E. coli* O22 and *E. coli* O91 were selected as target genes. The assays were serogroup-specific when tested against 72 *E. coli* O22 strains and 57 *E. coli* O91 strains isolated from food, humans, and animals, representative strains belonging to 168 *E. coli* O serogroups and non-*E. coli* bacteria. Furthermore, 72 *E. coli* O22 strains and 57 *E. coli* O91 strains isolated from food, water, animals, and humans were tested by the PCR for the presence of six and 19 virulence genes, respectively, associated with pathogenic *E. coli* strains. Based on the PCR screening results, multiplex PCR assays targeting the O22 *wzy* gene and the *cnf*-1 and *sfa* genes in *E. coli* O22 and the O91 *wzy* gene, conserved sequences of stx_1 and stx_2 genes, and the *astA* and *cdt*-III genes in *E. coli* O91 were developed to detect and identify pathogenic strains belonging to serogroups O22 and O91. Furthermore, *E. coli* O22 and O91 were detected by multiplex PCR assays targeting the *wzx* or *wzy* genes and conserved sequences of the stx_1 and stx_2 genes in ground beef samples inoculated with approximately two colony-forming units (CFU)/25 g after 18-h enrichment. The results demonstrate that the *E. coli* O22 and O91 *wzx* and *wzy* gene sequences were specific for the respective serogroups and can be used as diagnostic markers for rapid identification of these serogroups as an alternative to serotyping. The multiplex PCR assays targeting the O22 and O91 *wzx* and *wzy* genes and virulence genes can be used to identify and to detect pathogenic strains of these serogroups in food and fecal samples.

Keywords *E. coli* O91 · *E. coli* O22 · O-antigen · Detection · Identification · Multiplex PCR · Virulence Genes

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Introduction

In humans, pathogenic strains of *Escherichia coli* are responsible for intestinal diseases (gastroenteritis) and extraintestinal infections, which include urinary tract infections (UTI), bacteremia, and neonatal meningitis. *E coli* accounts for more than 90% of all uncomplicated UTIs. The diseases caused by *E. coli* depend on possession of specific virulence determinants. The pathogenic strains of *E. coli* that cause UTIs and other extraintestinal infections in humans are referred to as extraintestinal pathogenic *E. coli* (ExPEC). The interaction between different virulence factors allows ExPEC strains to colonize host mucosal

surfaces. The putative virulence factors of ExPEC include adhesins, siderophores, toxins, protectins, and invasins, some of which are encoded on pathogenicity-associated islands (Groisman and Ochman 1996; Kao et al. 1997). ExPEC bacteria express an array of structures, including P, S, Dr, and type-1 fimbriae, that are encoded by chromosomal gene clusters. A large prevalence of digalactoside binding P fimbriae (encoded by *pap*, pyelonephritis-associated pili), especially variant III of the digalactoside-specific P-fimbrial adhesin molecule, *papG* allele III, has been observed in ExPEC strains (Johnson et al. 2000). In addition to *pap*, human ExPEC-associated virulence genes include *sfa* (S fimbriae), *foc* (F1C fimbriae), *hly* (hemolysin), and cytotoxic necrotizing factor-1 (*cnf*-1; Johnson et al. 2001). *E. coli* O22 is one of the major serogroups associated with UTI in humans (Blanco et al. 1996a; Terai et al. 1997). Johnson et al (2005) isolated ExPEC, including strains that cause UTIs, from food products; therefore, it is possible that ExPEC may be food-borne pathogens. Shiga toxin-producing *E. coli* (STEC) strains belonging to serogroup O22 have also been isolated from animals and from humans with diarrhea or hemolytic uremic syndrome (HUS; http://www.microbionet.com.au/vtectable.htm; Bettelheim 2007).

Enterohemorrhagic *E. coli* serogroup O91 strains have caused non-bloody diarrhea, hemorrhagic colitis (HC), and HUS, and *E. coli* O91:H21 strains were shown to be virulent in an orally infected mouse model with a 50% lethal dose of less than ten bacteria (Lindgren et al. 1993; Beutin et al. 1998; Bonnet et al. 1998; Cantarelli et al. 2000). Virulence genes in pathogenic *E. coli* serotype O91 strains include those that encode the Shiga toxins (stx_1, stx_2), hemolysin (*ehxa*/*hlyA*), STEC autoagglutinating adhesion (*saa*), and intimin (*eae*; Beutin et al. 1998, 2004; Cid et al. 2001; Paton et al. 2001; Blanco et al. 2004).

Lipopolysaccharide is present on the outer membrane of Gram-negative bacteria and consists of three components: (1) lipid A; (2) an oligosaccharide core made of sugars and sugar derivatives, which links the lipid A to the O-antigen; and (3) a lateral polysaccharide O-antigen, which is responsible for antigenic specificity and determines the O serogroup. Genes for synthesis of the O-antigen are found in the O-antigen gene cluster, which is flanked upstream by a 39-bp JUMPstart sequence and downstream by the *gnd* gene (Hobbs and Reeves 1994; Reeves et al. 1996). The O-antigen cluster genes that encode for enzymes required for O-antigen synthesis vary among the different *E. coli* serogroups. The structural and antigenic variation of the O-antigens, therefore, is reflected by the genetic variation among the O-antigen gene clusters. Polymerase chain reaction (PCR) assays have been designed for identifying the different *E. coli* serogroups targeting unique gene sequences in the O-antigen gene clusters of the different serogroups (Beutin et al. 2005; DebRoy et al. 2004; Feng et al. 2004; Fratamico et al. 2003). The objectives of this study were the following: (1) to determine the DNA sequence of the *E. coli* O22 O-antigen gene cluster and identify the genes unique to this serogroup and (2) to develop multiplex PCR assays for detection of pathogenic *E. coli* O22 and O91 strains targeting virulence genes and genes in the respective O-antigen gene clusters. The DNA sequence of the *E. coli* O91 O-antigen gene cluster was reported by Perelle et al. (2002).

Materials and Methods

DNA Sequencing and Identification of Genes in the O-antigen Gene Cluster of *E. coli* O22

The O-antigen gene cluster of *E. coli* O22:H1 E14a was sequenced, and O22:H1 E14a and O91:H– H307b (Orskov et al. 1977) were used as reference control standards for development of PCR assays. Sequencing of the O-antigen gene cluster of *E. coli* O22 was performed as described previously by amplification of the gene cluster region by long PCR using primers targeting the JUMPstart and *gnd* regions, DNase I digestion of the long PCR product, cloning of products into the pGEM-T vector (Promega, Madison, WI, USA), and DNA sequencing using an Applied Biosystems 3700 automated DNA sequencer and the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA; Fratamico et al. 2003). The sequence data were analyzed using Sequencher software (Genecodes, Ann Arbor, MI, USA), and the assembled sequences were imported into Artemis (Rutherford et al. 2000), the open reading frames (ORFs) were located, and the putative coding regions were ascertained by analyzing the similarity with other published sequences in GenBank (Table 1).

Multiplex PCR Assays Targeting the *wzx* and *wzy* Genes

Multiplex PCR assays were developed for identification of *E. coli* serogroups O22 and O91 targeting the *wzx* and *wzy* genes found in the O-antigen gene clusters of both O serogroups. Template DNA for the PCR assays was prepared by mixing a colony from Luria–Bertani (LB) agar in sterile distilled water and heating at 100 °C for 20 min in a heating block. The suspension was centrifuged at 13,000×*g* for 5 min, and the supernatant containing the DNA was used for the PCR. PCR assays were developed using the primers listed in Tables 2 and 3, designed using the Primer3 software program, for amplification of regions within the *wzx* and *wzy* genes in the O-antigen gene clusters of *E. coli* O22 and O91. For the multiplex PCR assays targeting *wzx* and *wzy* and singleplex assays targeting *wzx* or *wzy*, reaction contents (11-μl total reaction volume) consisted of 3 μl of template DNA, 0.2 μM of each of the

Table 1 ORFs in the O-antigen gene cluster of *E. coli* serogroup O22

ORF no.	Proposed gene name	Location	No. of amino acids	Putative function	Most significant homology (accession no.)	% identity/ % similarity
1	*wclH*	56-1147	363	Glycosyl transferase	WbwZ [*Shigella boydii*] (AAY23341.1)	40/59
2	*wclI*	1144-1932	262	Glycosyl transferase, family 2	Glycosyl transferase, family 2 [*Prosthecochloris vibrioformis* DSM 265] (ZP_00661937.1)	35/56
3	*wclJ*	1929-2798	289	Glycosyl transferase	CheW protein [*Saccharophagus degradans* 2-40] (YP_527595.1)	26/48
4	*wzx*	2799-4034	411	Membrane spanning protein	hypothetical membrane spanning protein [*Propionibacterium acnes* KPA171202] (YP_054848.1)	23/44
5	*wclK*	4003-4587	194	Acetyltransferase	CheW protein [*Saccharophagus degradans* 2-40] (YP_527594.1)	50/68
6	*wclL*	4626-5744	372	Glycosyl transferases group 1	RfaG protein [*Pseudoalteromonas tunicata* D2] (ZP_01133499.1)	30/48
7	*wzy*	5731-6948	405	Polysaccharide biosynthesis protein	Amylovoran biosynthesis protein AmsL [*Sodalis glossinidius* str. '*morsitans*'] (YP_454656.1)	24/44
8	*galE*	6959-7975	338	UDP-glucose C4-epimerase	UDP-glucose C4-epimerase [*Escherichia coli*] (AAO37702.1)	61/76

primers (Integrated DNA Technologies Inc., Coralville, IA, USA), 0.18 mM of each of the four dNTPs, 2 mM $MgCl_2$ (for the O22 *wzx* and *wzy* PCR assays) or 3 mM $MgCl_2$ (for the O91 *wzx* and *wzy* PCR assays), 0.4 U of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA), 50 mM Tris (pH 8.3), 250 μg/ml bovine serum albumin, 2% sucrose, and 0.1 mM Cresol Red. The PCR was performed in a RapidCycler (Idaho Technologies Inc., Salt Lake City, UT, USA) using a Rapid-Cycle DNA amplification method (Wittwer et al. 1994), which consisted of 30 cycles of template denaturation at 94 °C, primer annealing at 58 °C for 0 s, and extension at 72 °C for 7 s for the *E. coli* O22 assays and annealing at 55 °C for 0 s and extension at 72 °C for 25 s for the *E. coli* O91 PCR assays. The amplification products were subjected to electrophoresis in 1% agarose gels at 200 V for 1 h, and the gels were stained with ethidium bromide and then visualized under UV light. Positive samples were identified based on the presence of bands of the expected sizes compared to results using DNA from O22 and O91 control standard reference strains.

Testing for Specificity of the PCR Assays

The *E. coli* O22 multiplex PCR assays were tested against 72 strains belonging to serogroup O22 isolated from animals, humans, food, and water in the last 25 years, and the *E. coli* O91 multiplex PCR assay was tested against 57 *E. coli* O91 strains isolated during the last 50 years from various sources. The specificity of the O22 and O91 multiplex PCR assays was determined using reference O standard strains that belonged to serogroups O1 to O175 except for O14, O31, O47, O67, O72, O93, O94, and O122 serogroups because these are not designated (Orskov et al. 1977). Fifty randomly selected cultures belonging to serogroups other than O22 or O91 were also tested for the presence of O22 *wzx* and O22 *wzy* and O91 *wzx* and *wzy*. Twenty other bacterial species were tested, and these included *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus aerogenes*, *Enterococcus faecalis*, *Hafnia alvei*, *Klebsiella pneumonia*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella enterica* subsp. Anatum, Arizona, Choleraesuis, Enteritidis, and Typhimurium, *Serratia marcescens*, *Shigella boydii*, *Staphylococus aureus*, *Vibrio cholerae*, and *Yersinia enterocolitica*. All of the bacteria tested were grown on LB agar at 37 °C.

Testing for the Presence of Virulence Genes in *E. coli* O22 and O91

E. coli O22 strains (n=72) were tested by the PCR for the presence of virulence genes associated with UTI. The primers (Table 2) and PCR cycling protocol targeting *cnf*-1

Table 2 Oligonucleotide primers for PCR assays for determination of presence of virulence factors and for detection of *E. coli* O22 by multiplex PCR

Target gene	Primer name and sequence 5′ to 3′	Size of PCR product	Reference
cnf-1[a]	CNF1-A F-GAACTTATTAAGGATAGT CNF1-B F-CATTATTTATAACGCTG	543	Blanco et al. 1996b
sfa[a]	sfa1 F-CTCCGGAGAACTGGGTGCATCTTAC sfa2 R-CGGAGGAGTAATTACAAACCTGGCA	410	Rodriguez-Siek et al. 2005
wzy[a]	O22wzy F-TTTACTGGCTGCTGCTACTGCT O22wzy R-TCATCTCCACCACGAGTCGAAAGT	246	This study
wzx	O22wzx F-TGTCGCCACTACTTTCCGCGTTTA O22wzx R-AGCCCATGACATTACTACGGCACT	458	This study
papG allele I	papG I F-TCGTGCTCAGGTCCGGAATTT papG I R-TGGCATCCCCCAACATTATCG	461	Rodriguez-Siek et al. 2005
papG allele III	papG III F-GGCCTGCAATGGATTTACCTGG papG III R-CCACCAAATGACCATGCCAGAC	258	Rodriguez-Siek et al. 2005
focG	focG F-CAGCACAGGCAGTGGATACGA focG R-GAATGTCGCCTGCCCATTGCT	364	Rodriguez-Siek et al. 2005
hlyA	hlyA F-AGCCGGAAGAGTTCTCTCAG hlyA R-CCAGCATAACAGCCGATGT	525	This study
16S rRNA	16SrRNA F-CCTCTTGCCATCGGATGTG 16SrRNA R-GGCTGGTCATCCTCTCAGACC	99	This study

[a] Primer sets used for multiplex PCR assays for *E. coli* O22

were described by Blanco et al. (1996b); targeting the *papG* alleles I and III was according to Johnson et al. (1998), and targeting *sfa* and *focG* was according to Rodriguez-Siek et al. (2005). Primers targeting the enterohemorrhagic *E. coli* *hlyA* gene (Table 2, nt 1374–1901 bp 525 amplicon size) were designed using the Primer3 program from the sequence reported in GenBank (accession no. AB032930). The PCR consisted of template DNA (3 μl), 0.50 μM of the two primers, and the rest of the protocol was as described above using 2 mM $MgCl_2$. Denaturation was carried out at 94 °C, annealing at 62 °C, and extension at 74 °C. In addition, *E. coli* O91 strains (n=57) were tested by the PCR for the presence of virulence genes associated with diarrheagenic *E. coli* (Table 3). The PCR primers and cycling protocols were as described in the references shown in Table 3. Primers targeting conserved sequences of stx_1 and stx_2 (305-bp product) were designed using the Primer3 program, and 0.25 μM of primers were used in the PCR. Laboratory positive control strains harboring the appropriate targeted gene were included in each of the PCR assays.

Multiplex PCR Assays Targeting *E. coli* O22 and O91 Virulence Genes and the *wzy* Gene

Multiplex PCR assays were developed for detection of *E. coli* O22 strains targeting the *E. coli* O22 *wzy* gene and the *sfa* (S fimbrial adhesin) and *cnf*-1 (cytotoxic necrotizing factor 1) genes using the primers shown in Table 2. Multiplex PCR assays were developed for detection of *E. coli* O91 targeting the *astA* (enteroaggregative heat stable toxin), *wzy*, and *cdt*-III (cytolethal distending toxin-III) genes and conserved sequences of the stx_1 and stx_2 genes using primers shown in Table 3. The multiplex PCR assays were performed using the QIAGEN multiplex PCR kit (Qiagen, Inc., Valencia, CA, USA) following the universal multiplex cycling protocol provided by the manufacturer and an annealing temperature of 57°C. Five microliters of template DNA was used in 50 μl of total reaction volume, which contained 0.25 μM of primers targeting *cnf*-1 and *sfa* and 0.5 μM of primers targeting the O22 *wzy* gene (Table 2) for the multiplex PCR for *E. coli* O22 and 0.1 μM of primers targeting *cdt*-III, 0.25 μM of primers targeting the O91 *wzy* gene and conserved sequences of stx_1 and stx_2 genes, and 0.5 μM of primers targeting the *astA* gene (Table 3) for the multiplex PCR for *E. coli* O91. The PCR products were separated by agarose gel (1.5%) electrophoresis containing 0.5 μg/ml ethidium bromide and then visualized under UV light and photographed.

Detection of *E. coli* O22 in Fecal Samples

To establish that the multiplex PCR assay could be used to detect *E. coli* O22 in environmental samples, dog feces (0.2 g) were spiked with *E. coli* O22:H1 E14a at 10^5 and 10^6 CFU concentrations. The samples were added to 2 ml of tryptic soy broth, mixed by vortexing for 1 min, and incubated at 37 °C for 6 h. DNA was extracted from 200 μl of the samples using the QIAamp DNA mini kit (Qiagen), and multiplex PCR assays were performed using the QIAGEN multiplex PCR kit as described above. Primers

Table 3 Oligonucleotide primers for PCR assays for determination of presence of virulence factors and for detection of *E. coli* O91 by multiplex PCR

Target gene	Primer name and sequences 5′ to 3′	Size of PCR product	Reference
stx_1/stx_2[a]	slt305 F-AAACGTAAGGCTTCAGCTGTGACAGT slt305 R-ATTGTTGAGCGAAATAATTTATATGTG	305	This study
stx_1	SLTI F-TGTAACTGGAAAGGTGGAGTATACA SLTI R-GCTATTCTGAGTCAACGAAAAATAAC	210	Fratamico et al. 2000
stx_2	SLTII F-GTTTTTCTTCGGTATCCTATTCC SLTII R-GATGCATCTCTGGTCATTGTATTAC	484	Fratamico et al. 2000
astA[a]	EAST1-1 F-CCATCAACACAGTATATC EAST1-2 R -GTCGCGAGTGACGGCTTTGT	111	Osek, 2003
wzy[a]	O91wzy F-CGCATTTAAGGACTGGCTGT O91wzy R-GTAGCAGATATGCCGACCGT	277	This study
wzx	O91wzx F-TTGCATCTGGCGCAATAAACACGG O91wzx R-ACACCATCCCAAATACCTGCTTGC	616	This study
cdt-I	CDT-Is F-CAATAGTCGCCCACAGGA CDT-Ias R-ATAATCAAGAACACCACCAC	411	Tóth et al. 2003
cdt-III[a]	CDT-IIIs F-GAAAGTAAATGGAATATAAATGTCCG CDT-IIIas R-TTTGTGTCGGTGCAGCAGGGAAAA	555	Tóth et al. 2003
cdt-IV	CDT-IVs F-CCTGATGGTTCAGGAGGCTGGTTC CDT-IVas R-TTGCTCCAGAATCTATACCT	350	Tóth et al. 2003
faeG (F4, K88)	F4-F-GGTGATTTCAATGGTTCGGTC F4ac-R-CCCAGCCGACGATTCAGAACCCCT	500	Kwon et al. 2002
fanA (F5, K99)	F5-F-TGCGACTACCAATGCTTCTG F5-R-TATCCACCATTAGACGGAGC	450	Kwon et al. 2002
fasS (F6, 987P)	F6-F-TCTGCTCTTAAAGCTACTGG F6-R-AACTCCACCGTTTGTATCAG	333	Kwon et al. 2002
fedA (F18, F107)	FedA-1-GTGAAAAGACTAGTGTTTATTTC FedA-2-CTTGTAAGTAACCGCGTAAGC	510	Kwon et al. 2002
$fimF_{41a}$ (F41)	F41-F-GAGGGACTTTCATCTTTTAG F41-R-AGTCCATTCCATTTATAGGC	431	Kwon et al. 2002
cnf-1	CNF1-A-GGCGACAAATGCAGTATTGCTTGG CNF1-B-GACGTTGGTTGCGGTAATTTTGGG	552	Pass et al. 2000
cnf-2	CNF2-A-GTGAGGCTCAACGAGATTATGCACTG CNF2-B-CCACGCTTCTTCTTCAGTTGTTCCTC	839	Pass et al. 2000
$eaeA_{GEN}$	EAE-F-GTGGCGAATACTGGCGAGACT EAE-R-CCCCATTCTTTTTCACCGTCG	890	Gannon et al. 1997
$hlyA_{933}$	hlyA-F-ACGATGTGGTTTATTCTGGA hlyA-R-CTTCACGTCACCATACATAT	166	Fratamico et al. 2000
elt (LT)	LTA-1-GGCGACAGATTATACCGTGC LTA-2-CCGAATTCTGTTATATATGTC	696	Blanco et al. 1997
bfp	bfp1-F-GATTGAATCTGCAATGGTGC bfp2-R-GGATTACTGTCCTCACATAT	597	Wieler et al. 1996
estIa (STa)	STa-1-TTAATAGCACCCGGTACAAGCAGG STa-2-CTTGACTCTTCAAAAGAGAAAATTAC	147	Blanco et al. 1997
estIb (STb)	STb-1-ATCGCATTTCTTCTTGCATC STb-2-GGGCGCCAAAGCATGCTCC	172	Blanco et al. 1997

[a] Primer sets used for multiplex PCR assays for *E. coli* O91 (Fig. 4). Primer set slt305F/slt305R was used for multiplex PCR assays targeting both *E. coli* O22 and *E. coli* O91 (Fig. 2b).

were used at a concentration of 0.25 μM. The multiplex PCR assay targeted the O22 *wzx* and *wzy* genes, and primers targeting an internal control 16S ribosomal RNA (rRNA) gene (Table 2), yielding a 99-bp product, were also included in each PCR assay. The primers for the *E. coli* 16S rRNA internal control were designed based on sequences in GenBank.

Detection of *E. coli* O22 and O91 in Ground Beef

Ground beef (20% fat) was purchased at a local supermarket. *E. coli* O22:H5 95-3322, which carries stx_2 (Centers for Disease Control and Prevention, Atlanta, GA) and *E. coli* O91:H21 96-1516 (Table 5) were inoculated into 25-g portions of the ground beef in sterile 500-ml volume flasks

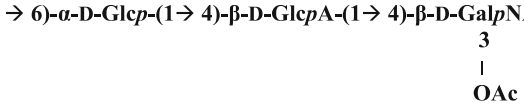

Fig. 1 Structure of the *E. coli* O22:K13 O-antigen (Bartelt et al. 1994)

at concentrations of approximately 2 and 20 CFU/25 g, and 225 ml of modified EC broth (Becton Dickinson, Sparks, MD, USA) containing 20 mg/l of novobiocin was added. The flasks were incubated at 42°C at 150 rpm for 18 h. One milliliter of the enrichments was centrifuged at 16,000×*g* for 2 min, and the DNA was extracted from the pellet using the PrepMan Ultra reagent (Applied Biosystems) according to the manufacturer's instructions. The multiplex PCR assays were performed using the QIAGEN multiplex PCR kit as described above, and all primers were used at a concentration of 0.25 μM. The multiplex PCR assays for *E. coli* O22 targeted the *E. coli* O22 *wzx* and *wzy* genes, conserved sequences of stx_1 and stx_2, and the internal control 16S rRNA sequence. For *E. coli* O91, the multiplex PCR assays targeted the *E. coli* O91 *wzy* gene, stx_1/stx_2, and the internal control.

Results and Discussion

DNA Sequencing and Analysis of the *E. coli* O22 O-antigen Gene Cluster

Analysis of the DNA sequence of the region containing the O22 O-antigen gene cluster showed that it contained eight complete ORFs, with all having the same transcriptional direction (GenBank accession no. DQ851855). 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* O22 cluster consists of genes encoding enzymes involved in sugar biosynthesis pathways, sugar transfer, and O-antigen processing. ORF8 is the only sugar biosynthesis gene present in the O22 O-antigen gene cluster, and it shows 76% similarity to a putative UDP-glucose C4-epimerase found in *E. coli* O128; therefore, it was named *galE*. Although the *galE* gene is a housekeeping gene that is usually found elsewhere on the chromosome (Samuel and Reeves 2003), this gene has been found in several O-antigen gene clusters, including O103, O113, and O128 (Fratamico et al. 2003; Paton and Paton 1999; Shao et al. 2003). The polysaccharide structure of *E. coli* O22 consists of 2-acetamido-2-deoxy-D-galactose, D-glucuronic acid, D-glucose, and D-galactose (Bartelt et al. 1994; Fig. 1). Since galactose, glucoronic acid, and glucose are all common sugars that can be used in many pathways, the genes for the biosynthesis of these sugars are housekeeping genes and are likely located elsewhere in the chromosome. Five sugar transferases were identified, which is consistent with the narrow substrate specificity of sugar transferases (Samuel and Reeves 2003). Since the O22 O-antigen is a pentasaccharide, ORF1-3 and ORF6 are likely responsible for sequentially transferring the precursor sugars to form the oligosaccharide on the undecaprenyl phosphate lipid carrier. ORF5 shows high homology to a number of acetyltransferases, and it may be involved in acetyl group transfer in the biosynthesis of 2-acetamido-2-deoxy-D-galactose. The O-antigen flippase gene (*wzx*, ORF4) transports the repeat sugar units across the cytoplasmic membrane, and the O-antigen polymerase gene (*wzy*, ORF7) polymerizes the repeat units. Analysis of the number of membrane-spanning regions of these two proteins was performed (Tusnády and Simon 1998, 2001). There were nine and 12 membrane spanning helices in the *E. coli* O22 Wzx and Wzy proteins, respectively.

Selection of Genes in the *E. coli* O22 and O91 O-antigen Gene Clusters for Development of Serogroup-Specific PCR Assays

The *wzx* and *wzy* genes, located between nucleotides 2799-4034 and 5731-6948, respectively, were selected as targets

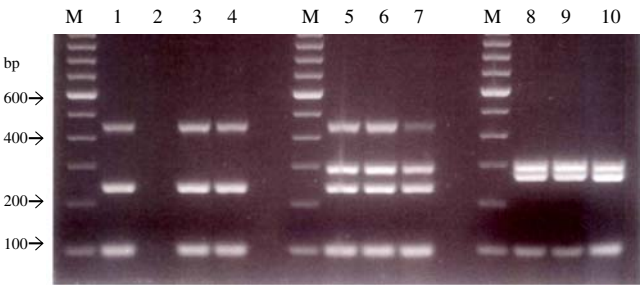

Fig. 2 Multiplex PCR detection of *E. coli* O22:H1 E14a targeting the *wzx* and *wzy* genes in spiked dog feces and ground beef. *Lane M*, molecular weight markers. *Lane 1*, PCR products for O22 *wzx* (458 bp) and O22 *wzy* (246 bp) from standard reference strain E14a (positive control) and 16S rRNA internal control (99 bp). *Lane 2*, H_2O (negative control). *Lane 3*, O22 *wzx* and *wzy* genes amplified from dog feces spiked with *E. coli* O22 at 10^5 CFU/g of dog feces. *Lane 4*, dog feces spiked with *E. coli* O22 at 10^6 CFU/g. *Lanes 5–10*, multiplex PCR results for detection of O22:H5 95-3322 and O91:H21 96.1516 in ground beef. *Lanes 5 and 6*, samples inoculated with *E. coli* O22:H5 at two CFU/25 g. *Lane 7*, sample inoculated with 20 CFU//25 g and all samples subjected to enrichment for 18 h at 42°°C (target genes: O22 *wzx*—458 bp, stx_1/stx_2—305 bp, O22 *wzy*—246 bp, and 16S rRNA internal control—99 bp). *Lanes 8 and 9*, samples inoculated with O91:H21 96.1516 at 2 CFU/25 g. *Lane 10*, sample inoculated with 20 CFU/25 g and subjected to enrichment for 18 h at 42°C (target genes: O91 *wzy*—277 bp, stx_1/stx_2—305 bp, and 16S rRNA internal control—99 bp)

Table 4 Presence of virulence genes in *E. coli* O22 strains isolated from different sources

Strain no.	Source	O22 *wzx*	O22 *wzy*	H type	*cnf*-1	*hlyA*	*papG* I/III	*sfa*	*focG*
79.0001	Cow	+	+						
79.0158	Cow	+	+	10					
79.0179	Cow	+	+	8					
79.0190	Chicken	+	+	8					
80.0346	Cow	+	+	8					
80.0537	Cow	+	+						
81.0236	Human	+	+	8				+	
81.0287	Human	+	+		+			+	
81.0517	Cow	+	+	10					
82.0313	Human	+	+	1	+			+	+
83.0201	Pig	+	+	6					
84.0278	?	+	+	1	+			+	
84.0314	?	+	+		+			+	+
84.0490	Chicken	+	+	2					
85.0708	Chicken	+	+	6					
85.0966	Dog	+	+		+		III+	+	+
85.1674	Dog	+	+		+			+	+
87.0771	Mouse	+	+	8	+				
87.1560	Horse	+	+	2					
87.1561	Horse	+	+	7					
88.0515	Human	+	+		+			+	+
88.0641	Cow	+	+	21					
88.1045	Horse	+	+	7					
88.1410	Chicken	+	+	11					
88.1411	Chicken	+	+	11					
88.1412	Chicken	+	+	11					
88.1414	Chicken	+	+	11					
88.1427	Chicken	+	+	11					
88.1428	Chicken	+	+						
90.0384	Cow	+	+	11		+		+	
90.2091	Human	+	+		+			+	
90.2539	Chicken	+	+	2					
90.2562	Chicken	+	+	2					
90.2566	Chicken	+	+						
90.2627	Chicken	+	+						
90.2700	Chicken	+	+						
92.1032	Human	+	+		+		III+	+	
94.0215	Human	+	+	1	+		III+	+	+
94.0216	Human	+	+		+		III+	+	+
95.0031	Chicken	+	+						
95.0370	Food	+	+	2		+			
95.4378	Chicken	+	+						
96.0599	?	+	+	8					
97.0165	Human	+	+	7	+			+	
97.1634	Dog	+	+					+	+
98.0318	Turkey	+	+						
98.0321	Turkey	+	+						
98.0325	Turkey	+	+						
99.0698	Turkey	+	+			+			
99.1100	Dog	+	+					+	+
99.1845	Dog	+	+					+	+
0.0780	Chicken	+	+						
0.0790	Chicken	+	+						
0.2222	Turkey	+	+						
0.2389	Turkey	+	+						
1.0238	Human	+	+		+			+	+

Table 4 (continued)

Strain no.	Source	O22 *wzx*	O22 *wzy*	H type	*cnf*-1	*hlyA*	*papG* I/III	*sfa*	*focG*
1.0546	Human	+	+					+	
1.0791	?	+	+			+			
1.0912	?	+	+			+			
1.0913	?	+	+			+			
1.0914	?	+	+			+			
1.0915	?	+	+			+			
1.0918	?	+	+			+			
1.2359	?	+	+		+			+	
1.4110	Human	+	+		+		I+	+	
1.4236	Human	+	+					+	+
2.3284	?	+	+					+	+
3.2150	?	+	+		+			+	
3.2853	Water	+	+						
3.3068	?	+	+		+			+	+
3.3078	Water	+	+					+	+
3.3216	Water	+	+	1	+			+	+
% positive[a]		100	100		26	13	7[b]	38	22

[a] Percentage of strains positive for the different virulence out of the total number of strains tested (72)
[b] One percent of the strains was positive for *papG I*, and 6% were positive for the *papG III* allele.

for PCR assay development. In addition, the *E. coli* O91 *wzx* and *wzy* genes, located between nucleotides 4847-6112 and 8912-10113, respectively (GenBank accession no. AY035396; Perelle et al. 2002) were also targeted for PCR assay development. Due to the relatively low similarity in Wzx and Wzy among different *E. coli* serogroups, the genes coding for these enzymes have been found to be suitable targets for serogroup-specific PCR assay development (Beutin et al. 2005; DebRoy et al. 2004; Feng et al. 2004; Fratamico et al. 2003), and likewise, there was low percent identity of the O22 *wzx* and *wzy* genes to other sequences in GenBank. Therefore, these genes in *E. coli* O22 and O91 were selected for developing *E. coli* O22 and O91 serogroup-specific multiplex PCR assays. All of the isolates belonging to serogroups O22 and O91 exhibited the presence of the respective *wzx* and *wzy* genes showing the reliability of the multiplex assays. None of the non-O22 and non-O91 *E. coli* serogroups and non-*E. coli* bacteria tested by the multiplex PCR assays was positive for the O22 or O91 *wzx* and *wzy* genes; therefore, the multiplex PCR assays were specific and are useful for identifying *E. coli* serogroups O22 and O91.

Detection of *E. coli* O22 in Dog Feces

E. coli O22 was detected by multiplex (targeting both the O22 *wzx* and *wzy* genes) or singleplex (targeting either the O22 *wzx* or *wzy* gene) PCR when inoculated at both 10^5 and 10^6 CFU/0.2 g of dog feces. Using the multiplex PCR, bands of 458 (O22 *wzx*) and 246 (O22 *wzy*) bp were visible using DNA extracted from fecal samples inoculated with 10^5 and 10^6 CFU/02 g (Fig. 2). The product for the internal control 16S rRNA sequence was also visible in each lane.

Detection of STEC *E. coli* O22 and O91 in Ground Beef Targeting *wzx*, *wzy*, and Conserved Sequences of stx_1 and stx_2

Shiga toxin-producing *E. coli* O22:H5 95-3322 and *E. coli* O91:H21 96-1516 were detected by multiplex PCR assays targeting the *wzx* and *wzy* gene sequences of the respective serogroups and conserved sequences of the stx_1 and stx_2 genes. The target genes of both *E. coli* O22:H5 and O91:H21 were detected in ground beef enrichments inoculated

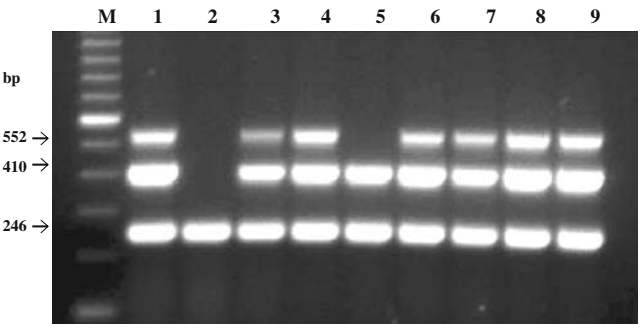

Fig. 3 Agarose gel showing results of multiplex PCR assay for detection of ExPEC *E. coli* O22 targeting the *sfa* (410 bp), *E. coli* O22 *wzy* (246 bp), and *cnf*-1 (552 bp) genes. *Lane M*, molecular weight standards, 100-bp DNA ladder; *lane 1*, *E. coli* O22 97.0165; *lane 2*, *E. coli* 95.0370; *lane 3*, *E. coli* 1.4110; *lane 4*, *E. coli* 90.2091; *lane 5*, *E. coli* 1.4236; *lane 6*, *E. coli* 1.0238; *lane 7*, *E. coli* 92.1032; *lane 8*, *E. coli* 94.0215; *lane 9*, *E. coli* 94.0216

Table 5 Presence of virulence genes in *E. coli* O91 strains isolated from different sources

Strain[a]	Source	O91 *wzx*	O91 *wzy*	H type	stx_1	stx_2	*fanA*	*astA*	*cdt*-I	*cdt*-III
65.0001	Water	+	+							
66.0147	Water	+	+	8						
66.0174	Water	+	+	7						
67.0073	Water	+	+	9						
67.0108	?	+	+							
68.0001	Water	+	+	28						
78.0153	Cow	+	+	5						
79.0286	Cow	+	+	28						
80.0437	Pig	+	+	10				+		
81.0070	Cow	+	+	10				+		
81.1238	Chicken	+	+							
83.1443	Chicken	+	+	28						
87.0004	Mouse	+	+	21						
87.1078	Turkey	+	+							
89.0628	Chicken	+	+	28						
90.0225	Turkey	+	+	34						
90.0725	Chicken	+	+	31						
90.1347	Chicken	+	+	21						
90.1885	Cow	+	+	8				+		
91.1577	Cow	+	+	10				+		
92.0485	Cow	+	+	7						
93.1060	Human	+	+	8						
94.0019	Human	+	+	21						
95.3947	?	+	+	10						
95.4521	Water	+	+	21						
96.0348	Pig	+	+	14	+					
96.0497	Human	+	+			+				+
96.1516	Human	+	+	21		+				+
97.0309	Chicken	+	+	19						
97.1088	Mink	+	+	28				+		
97.1424	Cow	+	+	10						
97.1913	?	+	+						+	
97.2084	Water	+	+	7						
98.0193	Turkey	+	+	45				+		
98.0339	Turkey	+	+	14						
99.0741	Food	+	+	7	+					
99.1451	Water	+	+	7						
99.1706	Chicken	+	+	34						
99.1859	Dog	+	+	34			+			
0.1242	Gazelle	+	+	36						
0.1367	Cow	+	+	29						
0.2404	Turkey	+	+	28						
0.2689	Sheep	+	+	14	+					
1.0606	Goose	+	+	36		+				+
1.0709	Goose	+	+	36	+					
1.0904	?	+	+	36		+				+
1.2190	Water	+	+	5				+		
1.3125	Pig	+	+	14						
1.3275	Water	+	+	5						
1.4657	?	+	+	14						
2.0739	Environment	+	+	21						+
2.2215	Food	+	+	21				+		
2.2971	?	+	+	7						
2.3209	?	+	+	21		+				+
3.3159	Dog	+	+	7						
3.4784	Goose	+	+	36						
4.0221	Water	+	+	14						
% positive[b]		100	100		7	9	2	14	2	11

[a] All strains were negative by the PCR for *faeG*, *fasA*, *fedA*, fim_{F41a}, *bfp*, *estIa*, *estIb*, *elt*, $eaeA_{GEN}$, *cdt*-IV, *cnf*-1, and *cnf*-2.
[b] Percentage of strains positive for the different virulence genes out of the total number of strains tested (57)

with either 2 or 20 CFU/25 g after 18 h of enrichment (Fig. 2). The product for the internal control 16S rRNA sequence was also visible in each lane.

Multiplex PCR Assays Targeting *E. coli* O22 and O91 Virulence Genes and the *wzy* Gene

E. coli strains belonging to serogroup O22 have been identified as ExPEC and a cause of urinary tract infections. Virulence genes, including *pap*, *sfa*, *afaI* (afimbrial adhesin-I), *aer* (aerobactin), *hlyA*, and *cnf*-1 are found in uropathogenic strains (Blanco et al. 1996a; Terai et al. 1997; Johnson 1991). *E. coli* serogroup O91 has been associated with cases of HC and HUS; therefore, this serogroup is classified as an enterohemorrhagic *E. coli* (Lindgren et al. 1993; Beutin et al. 1998; Bonnet et al. 1998; Keskimäki et al. 1998). However, not all strains of *E. coli* O22 and O91 are pathogens, since they may lack important virulence genes that would render them pathogenic. To identify potentially pathogenic ExPEC O22 and STEC O91, multiplex PCR assays targeting the *E. coli* O22 and O91 *wzy* gene and virulence genes were developed for detection and identification of these strains. To determine which genes were suitable targets for multiplex PCR development, virulence genes harbored by 72 *E. coli* O22 strains of different H types and isolated from various sources were tested by the PCR for the presence of six virulence genes associated with strains that cause UTI. Twenty-two percent, 13%, 6%, and 1% of the strains were positive for the *focG*, *hlyA*, *papGIII*, and *papGI* genes, respectively (Table 4). Also, 26% and 38% of the strains were positive for the *cnf*-1 and *sfa* genes, respectively. The *cnf*-1 and *sfa* genes were found in all O22 strains isolated from humans (12/72 strains tested), except for two strains that were negative for *cnf*-1. Based on the PCR screening results, a multiplex PCR assay was designed targeting the O22 *wzy* gene and the *cnf*-1 and *sfa* genes. Multiplex PCR results using DNA from several *E. coli* O22 strains harboring *sfa* and *cnf*-1 or only *sfa* are shown in Fig. 3. *E. coli* O22 95.0370 lacked *sfa* and *cnf*-1, and *E. coli* O22 1.4236 lacked a product for *cnf*-1. All of the O22 strains were positive for the *E. coli* O22 *wzy* gene.

In a similar manner, 57 *E. coli* O91 strains isolated from humans, animals, food, and the environment were tested for the presence of 19 virulence genes associated with diarrheagenic *E. coli.* Two percent of the strains were positive for the *fanA* and *cdt*-I genes (Table 5). Also, 7%, 9%, 14%, and 11% of the strains were positive for the stx_1, stx_2, *astA*, and *cdt*-III genes, respectively. The EAST1 enterotoxin, encoded by *astA*, has homology to the heat-stable enterotoxin of enterotoxigenic *E. coli.* This toxin was first identified in enteroaggregative *E. coli*; however, it is produced by other categories of diarrheagenic *E. coli*, as well, including

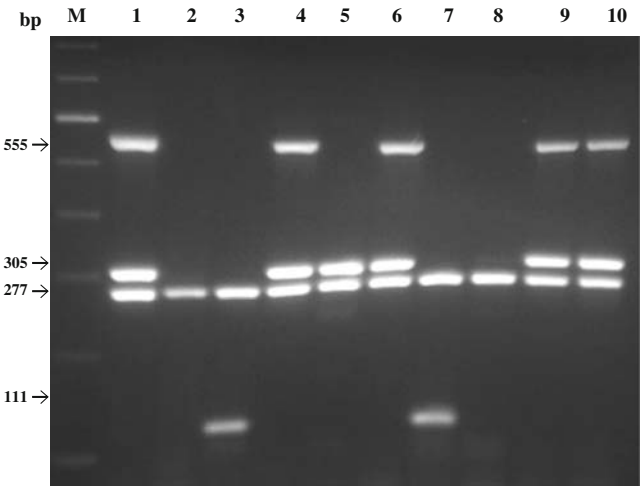

Fig. 4 Agarose gel showing results of multiplex PCR assay for detection of STEC *E. coli* O91 targeting the *astA* (111 bp), *E. coli* O91 *wzy* (277 bp), stx_1/stx_2 (305 bp), and *cdt*-III (555 bp) genes. *Lane M*, molecular weight standards, 100-bp DNA ladder; *lane 1*, *E. coli* O91 96.1516; *lane 2*, *E. coli* 87.0004; *lane 3*, *E. coli* 90.1885; *lane 4*, *E. coli* 1.0709; *lane 5*, *E. coli* 0.2689; *lane 6*, *E. coli* 1.0904; *lane 7*, *E. coli* 1.2190; *lane 8*, *E. coli* 3.3159; *lane 9*, *E. coli* 1.0606; *lane 10*, *E. coli* 2.3209

enterohemorrhagic and other Shiga toxin-producing *E. coli* strains. Cytolethal distending toxins (encoded by *cdt*-I–IV and the recently identified *cdt*-V genes) are produced by diarrheagenic *E. coli* as well as several other bacterial genera (Tóth et al. 2003; Oloomi and Bouzari 2006). We used PCR assays targeting the *cdt*-II gene and were unsuccessful at amplifying a product of the expected size using a *cdt*-II reference strain and using primers designed based on the *cdt*-II gene sequence (GenBank accession no. U04208) or two primer sets described by Oloomi and Bouzari (2006). These investigators also reported difficulties in identifying Cdt-II-producing strains by the PCR (Oloomi and Bouzari 2006). Therefore, in the current study, the most common virulence genes found in the O91 strains tested, which included strains isolated from humans, were stx_1, stx_2, *astA*, and *cdt*-III. Based on the PCR results, a multiplex PCR assay targeting the O91 *wzy* gene and conserved sequences of stx_1 and stx_2 and the *astA* and *cdt*-III genes in *E. coli* O91 was developed. Multiplex PCR results using DNA from several *E. coli* O91 strains harboring stx_1/stx_2, *astA*, and/or *cdt*-III are shown in Fig. 4. *E. coli* O91 strains 87.0004, 90.1885, 1.2190, and 3.3159 lacked a product for stx_1/stx_2; therefore, these strains were likely not STEC. All of the O91 strains were positive for the *E. coli* O91 *wzy* gene.

Conclusions

In the current study, the O-antigen gene cluster of an *E. coli* serogroup O22 strain was sequenced and the genes in the

cluster identified. PCR assays targeting the *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes in the O-antigen gene clusters of *E. coli* O22 and *E. coli* O91 were found to be specific for identification of the respective serogroups. Therefore, these genes can be used as diagnostic markers for rapid identification of these serogroups by PCR as an alternative to identification using antisera. Furthermore, the multiplex PCR assays targeting the O22 and O91 *wzy* genes and virulence genes can be used to identify and detect pathogenic strains of these two serogroups potentially in food and environmental samples and clinical specimens. Multiplex PCR assays targeting the *E. coli* O22 or O91 *wzx* and/or *wzy* and other virulence genes can also potentially be developed.

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