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Development of PCR Assays Targeting Genes in O-Antigen Gene Clusters for Detection and Identification of *Escherichia coli* O45 and O55 Serogroups

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The *Escherichia coli* O45 O-antigen gene cluster of strain O45:H2 96-3285 was sequenced, and conventional (singleplex), multiplex, and real-time PCR assays were designed to amplify regions in the *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes. In addition, PCR assays targeting the *E. coli* O55 *wzx* and *wzy* genes were designed based on previously published sequences. PCR assays targeting *E. coli* O45 showed 100% specificity for this serogroup, whereas by PCR assays specific for *E. coli* O55, 97/102 strains serotyped as *E. coli* O55 were positive for *wzx* and 98/102 for *wzy*. Multiplex PCR assays targeting the *E. coli* O45 and the *E. coli* O55 *wzx* and *wzy* genes were used to detect the organisms in fecal samples spiked at levels of 10^6 and 10^8 CFU/0.2 g feces. Thus, the PCR assays can be used to detect and identify *E. coli* serogroups O45 and O55.

Strains of *Escherichia coli* belonging to serogroup O45 have been isolated from animals and humans and classified as both enterotoxigenic *E. coli* and Shiga toxin-producing enterohemorrhagic *E. coli* (4, 16, 25). In addition, *E. coli* O45 strains isolated from diarrheic dairy calves produced cytotoxic necrotizing factor and were designated as strains of necrotoxigenic *E. coli* (10). Many *E. coli* O45 strains isolated from swine have been associated with postweaning diarrhea and demonstrated the attaching and effacing (A/E) phenotype, allowing them to adhere to intestinal epithelial cells (3). Six *E. coli* serogroups, including O45, were associated with feral pigeons and produced a variant of Shiga toxin 2 called Stx2f (9).

E. coli strains belonging to serogroup O55 have been classified as human enteropathogenic strains and are believed to be genetically related to enterohemorrhagic *E. coli* O157:H7, shown to have been derived from an O55:H7 ancestral clone (15, 23). Because of the potential pathogenicity of *E. coli* isolates belonging to serogroups O45 and O55, rapid and reliable assays for detecting and identifying these serogroups in food, environmental, and clinical samples are needed.

Conventionally, antigenic analysis of the ca. 179 different O serogroups in *E. coli* is performed by agglutination reactions using antisera raised in rabbits against the O standard reference strains. O serotyping is laborious, and cross-reactions between different serogroups often occur, giving equivocal results. Furthermore, *E. coli* strains occasionally undergo transition from smooth to rough forms as a result of mutations in one or more of the multiple genes controlling synthesis and polymerization of the O antigen. Rough isolates do not produce an O antigen and therefore cannot be typed by using antisera. In view of these facts, there is a need for the development of alternative methods for identifying and typing *E. coli* serogroups. Lipopolysaccharides (LPS) are essential components of the gram-negative bacterial outer membrane. They are composed of three parts: lipid A, which is composed of sugars and fatty acids and anchors the LPS in the outer membrane through covalent linkages; an oligosaccharide core made of sugars and sugar derivatives; and a lateral polysaccharide chain (O antigen) responsible for the antigenic specificity of the smooth form of each of the *E. coli* serogroups. The O antigen contains many repeats (often 10 to 30) of an oligosaccharide unit (O unit) generally composed of three to six sugars. Different combinations of sugars in the O unit, as well as diversity of the chemical linkages between the sugars, determine the diversity of the O antigens. Further levels of variation are conferred by the addition of nonsugar moieties (such as *O*-acetyl residues or amino acids) and by differences in the modal length of the polysaccharide chains (13).

In *E. coli*, genes encoding the enzymes involved in O-antigen synthesis are clustered in a chromosomal region referred to as the O-antigen gene cluster (formerly known as the *rfb* cluster), which is generally found between the *galF* gene and the *gnd* gene that encodes the housekeeping enzyme, 6-phosphogluconate dehydrogenase, involved in the pentose phosphate pathway (1, 2, 13). Upstream of the O-antigen gene cluster, there is a highly conserved 39-bp element called the JUMPstart sequence (8). The number of genes in the clusters varies depending on the complexity of the polysaccharide, and strains of different serogroups can show completely different gene sets. PCR-based tests amplifying certain genes in the *E. coli* O-antigen gene clusters have been found to be serogroup specific (5, 6, 19–22). The objective of this study was to develop rapid and specific PCR assays for detecting and typing *E. coli* serogroups O45 and O55 by targeting the *wzx* and *wzy* genes of the respective O-antigen gene clusters.

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The bacterial strains used in the study were from the culture collection of the Gastroenteric Disease Center at The Pennsylvania State University. *E. coli* O45:H2 strain 96-3285 (Centers for Disease Control and Prevention, Atlanta, GA), which was used for DNA sequencing of the O-antigen gene cluster, and *E. coli* O55:H6 strain Su 3972-41 (World Health Organization) (12) were used for the development of the PCR assays. The other reference standard strains used belonged to serogroups O1 through O175 with the exceptions of serogroups O31, O47, O72, O93, O94, and O122, which are not designated (12). Fifty-seven strains belonging to *E. coli* O45, 117 strains belonging to *E. coli* O55, and 47 non-O45 and non-O55 strains isolated from animals, humans, and the environment from the reference collection at the Gastroenteric Disease Center were used for determining the specificity of the assays. Other bacteria ($n = 21$) tested included *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella enterica* serovars Enterica, Arizonae, Choleraesuis, Enteritidis, and Typhimurium, *Serratia marcescens*, *Staphylococcus aureus*, *Shigella boydii*, *Vibrio cholerae*, and *Yersinia enterocolitica*. All bacteria were grown in Luria-Bertani (LB) broth or on LB agar plates.

Sequencing of the O-antigen gene cluster of *E. coli* O45:H2 was performed as previously described following amplification of the cluster by long PCR using primers targeting the JUMPstart and *gnd* regions, DNase I digestion, and cloning (6). The assembled sequences were imported into Artemis (14), the open reading frames (ORFs) were located (a cutoff of 150 was used to determine ORFs), and the putative coding regions were ascertained by analyzing the similarity with other published sequences in GenBank. Analysis of the DNA sequence of the 14,483-bp region containing the O45 O-antigen gene cluster showed that it contained 13 complete ORFs, with all having the same transcriptional direction. The genes within the cluster, identified with various degrees of precision and named in accordance with the system proposed by Reeves et al. (13), are shown in Table 1. The *E. coli* O45 cluster consisted of genes involved in sugar biosynthesis pathways, sugar transferase genes, and O-antigen-processing genes, including the O-antigen flippase gene (*wzx*) that transports the repeat sugar units across the cytoplasmic membrane and the O-antigen polymerase gene (*wzy*) that polymerizes the repeat units. The transmembrane regions of the proteins were analyzed as described previously (17, 18). The *wzx* and *wzy* genes, located between nucleotides 7144 and 8403 and nucleotides 9366 and 10514, respectively, were selected as targets for PCR assay development. In addition, the *E. coli* O55 *wzx* and *wzy* genes (GenBank accession number AF461121) (21) 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 are suitable targets for serogroup-specific PCR assay development (5, 6, 19, 20, 21)

Template DNA for the PCR assays was prepared by mixing a colony from the LB agar in sterile distilled water and heating at 100°C for 20 min in a heating block. The suspension was centrifuged at $13,000 \times g$ for 5 min, and the supernatant containing the DNA was used for the PCR. PCR assays were developed by using the primers listed in Table 2, designed by using the Primer3 software program, for amplifications of regions within the *wzx* and *wzy* genes in the O-antigen gene clusters of *E. coli* O45 and O55 (21). For the singleplex (*wzx* or *wzy*) PCR assays, each of the primer sets shown in Table 2 was used separately in the PCRs, and reaction mix contents for each PCR (11-μl total reaction mix volume) consisted of 3 μl of template DNA, 0.5 μM of primers (Integrated DNA Technologies Inc., Coralville, IA), 0.18 mM concentration of each of the four deoxynucleoside triphosphates, 2 mM $MgCl_2$ (for the O55 *wzx*, O45 *wzx*, and O45 *wzy* PCR assays) and 3 mM $MgCl_2$ (for the O55 *wzy* PCR assays), 0.4 U of *Taq* DNA polymerase (PGC Scientific, Gaithersburg, MD), 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) by using a rapid-cycle DNA amplification method (24) and consisted of 30 cycles of template denaturation at 94°C, primer annealing and primer extension at temperatures and times indicated in Table 3. The amplification products were subjected to electrophoresis in 1% agarose gels at 200 V for 1 h for all assays except for the O55 *wzy* PCR that was analyzed using a 2% gel. The gels were stained with ethidium bromide and visualized under UV light. Positive samples were identified based on the presence of bands of the expected sizes compared to results with O45 and O55 control standard reference strains.

A multiplex PCR assay targeting the *E. coli* O45 *wzx* and *wzy* genes (Fig. 1) was performed by using primers O45*wzx*2 and O45*wzy*2 (Table 2) at 20 μM concentrations for amplifying O45 *wzx*, following the thermocycling conditions presented in Table 3. A multiplex PCR assay targeting the *E. coli* O55 *wzx* and *wzy* genes (Fig. 1) was performed using primers O55*wzx*2 and O55*wzy*1 (Table 2) at 50 μM and 20 μM concentrations, respectively, according to the PCR conditions listed in Table 3. To establish that the multiplex PCR assay could be used to detect *E. coli* O45 or O55 in environmental samples, chicken feces (0.2 g) were spiked with *E. coli* belonging to either serogroup O45 or O55 at 10^6 and 10^8 CFU concentrations. The fecal matter was vortexed until homogeneous, and the DNA was extracted from the samples using the QIAamp DNA stool mini kit (QIAGEN, Inc.). Multiplex PCR assays were performed, and the results are shown in Fig. 1. Multiplex PCRs targeting O45 *wzx* and O55 *wzx* genes were also performed (data not shown) for rapid detection of these serogroups by use of primer pairs for O45 *wzx2* and O55 *wzx2* (Table 2) using the PCR conditions described in Table 3. Multiplex PCRs were as sensitive and specific as singleplex PCR.

TaqMan-based real-time PCR assays were also developed for detection of *E. coli* O45 and O55 targeting the *wzx* and *wzy* genes. Because the assay allows the detection of pathogens in real time during DNA amplification, this method is faster than conventional PCR and is advantageous for diagnostic purposes. Primers and probes were designed based on DNA sequences of the *E. coli* O45 *wzx* and *wzy* genes determined in the current study and on the O55 *wzx* and *wzy* genes as described previously (21). The TaqMan probes were labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM), at the 5′ end and a quencher dye, BHQ1, at the 3′ end (Biosearch Technologies Inc., CA). The primers and probes used for the assays are depicted in Table 2. The real-time PCR assays were performed using the ABI PRISM 7700 sequence detection

TABLE 1. Genes in the O-antigen gene cluster of *E. coli* serogroup O45

ORF no.	Proposed gene name	Location (nucleotides)	No. of amino acids in gene product	Putative function	Most significant homolog(s) (accession no.)	% Identity/ % similarity
1	*rmlB*	59–1198	379	dTDP-D-glucose-4,6-dehydratase	RmlB, dTDP-glucose 4,6-dehydratase, *Salmonella enterica* (AAG09513)	83/87
2	*wbhP*	1246–2196	316	NAD-dependent epimerase/dehydratase	ORF_12; similar to NAD-dependent epimerase/dehydratase family, *Pseudomonas aeruginosa* (AAM27579)	42/58
3	*wbhQ*	2196–3230	344	Glycosyltransferase	Probable *N*-acetylgalatosaminyl transferase, trsF (S51265), and WbcO, *Yersinia enterocolitica* (CAA87703)	50/68
4	*wbhR*	3231–3791	186	Acetyltransferase	Acetyltransferase, *Oceanobacillus iheyensis* (NP_693813) (BAC14847)	37/58
5	*wbhS*	3830–5716	628	TrsG protein homolog, role in galactose modification	TrsG protein homolog (T44517), ORF9P (BAA85014), and WbgZ (AAG17416), *Plesiomonas shigelloides*	61/79
6	*rmlC*	5797–6336	179	dTDP-4-dehydrorhamnose-3,5-epimerase	Putative dTDP-4-dehydrorhamnose-3,5-epimerase, *Aeromonas hydrophila* (AAM22546)	66/80
7	*wbhT*	6333–7151	272	dTDP-glucose-4,6-dehydratase	Putative dTDP-glucose-4,6-dehydratase, *Aeromonas hydrophila* (AAM22547)	55/69
8	*wzx*	7144–8403	419	O-antigen flippase	Membrane protein involved in the export of O-antigen and teichoic acid, 12 transmembrane domains, *Burkholderia fungorum* (ZP_00278649)	25/44
9	*wbhU*	8387–9352	321	Glycosyltransferase	Putative glycosyltransferase, *Pyrococcus furiosus* (NP_579088)	29/49
10	*wzy*	9366–10514	382	O-antigen polymerase	Transmembrane protein, 9 transmembrane domains, *Ralstonia solanacearum* (NP_519421.1)	27/45
11	*wbhV*	10507–11043	178	Serine acetyltransferase	Putative acetyltransferase, *Clostridium thermocellum* (ZP_00313397.1)	37/60
12	*wbhW*	11045–12055	336	Unknown	Eps11, *Streptococcus thermophilus* (AAN63793)	30/49
13	*rmlA*	12102–12974	290	Glucose-1-phosphate thymidylyltransferase	RmlA, *Raoultella terrigena* (AAQ82933.1)	78/89

TABLE 2. Oligonucleotide primers and probes

Target gene	Sequence (5′ to 3′)[a]	Location in cluster (nucleotides)	Amplicon size (bp)	Accession no. or source
Singleplex and multiplex PCR				
O45 *wzx1*	(F) CCG GGT TTC GAT TTG TGA AGG TTG	7769–8295	527	This study
	(R) CAC AAC AGC CAC TAC TAG GCA GAA			
O45 *wzx2*	(F) TAT GAC AGG CAC ATG GAT CTG TGG	7347–7601	255	
	(R) TTG AGA CGA GCC TGG CTT TGA TAC			
O45 *wzy1*	(F) GAA ATT ATG CCA TCT TGG CGA GCG	9570–10066	497	This study
	(R) CAT GTG AAG CCT GAA GGC AAA CTC			
O45 *wzy2*	(F) CTG ATG TCA GGC CTC GTG GAA ATA	9858–10308	451	
	(R) ATG TAA CCA CAA TAA GGG AGC CCG			
O55 *wzx1*	(F) AAT GGA ACA TTG CAA CAG CA	10746–10895	150	AF461121
	(R) TGT GGA TTC CAG AAA AGC AA			
O55 *wzx2*	(F) TCT TGT AAC TAA GTG GCC ACA GGC	10823–11505	683	
	(R) ATA ACA CCC AAC CTA TAC CTC CCG			
O55 *wzy1*	(F) GTG GTT TTG ACG ACT CGC TT	9380–9526	147	AF461121
	(R) CCA AAA AGC CCT GCA ACT AA			
Real-time PCR				
O45 *wzx*	(F) CGT TGT GCA TGG TGG CAT	7472–7543	72	This study
	(R) TGG CCA AAC CAA CTA TGA ACT G			
O45 *wzy*	(F) GGT GCT TTG TGA TAA TTC CTG ATG	9616–9691	76	This study
	(R) TTA TAG CCG CCC CTA AAT TGC			
O45 *wzx* probe	6-FAM d(ATT TTT TGC TGC AAG TGG GCT GTC CA)BHQ-1	7494–7519		This study
O45 *wzy* probe	6-FAM d(TTG CTG CTG GCG GGA TAC CAA TGA T)BHQ-1	9643–9667		This study
O55 *wzx*	(F) AAT TAA CGA ACA TAA CAC CCA ACC	11416–11516	101	AF461121
	(R) ATA TCT CTT CGT TAC TGT GTG TAT TTC			
O55 *wzy*	(F) AGC TTT CCT GGC GGG TTT	10118–10204	87	AF461121
	(R) GCA CCA CGC TAT CTT TTT TCT TAA T			
O55 *wzx* probe	6-FAM d(ACC TCC CGC TAA AAC CCC AAC TCT AGT AG)BHQ-1	11461–11489		AF461121
O55 *wzy* probe	6-FAM d(CCG CGG CGA TAT TGG GTA CTG C)BHQ-1	10142–10163		

[a] F, forward; R, reverse.

system. The reaction mix contained 25 μl of Universal Master Mix (P/N 4304437; Applied Biosciences Inc.), 2 μl of each primer (10 μM), 8 μl of probe (1 μM), and 42 μl of water. DNA from pure cultures was purified by using the QIAquick PCR purification kit (QIAGEN Inc., CA) by following the manufacturer's protocol. DNA (8 μl) was added to the mix, and the tubes were subjected to 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The tubes were held at 25°C for 2 min, and the fluorescence generated by the sequence-specific probes was measured. The ΔR_n is the fluorescence signal increase due to template amplification. The amplification plots were generated with the ΔR_n mean value on the *y* axis and the cycle number on the *x* axis (7). The threshold cycle is the cycle number at which the reporter fluorescence generated by the cleavage of the probe passes a fixed threshold value above baseline. A standard deviation of 10 units above baseline was used to determine the fixed threshold. The real-time PCR assays developed for *E. coli* O45 reproducibly showed a detectable fluorescence signal above the threshold at 14 cycles for 10^7 copies of *wzx* and *wzy*, and for *E. coli* O55, fluorescence above threshold was generated at 13 cycles for *wzx* at 10^8 copies of DNA and at 15 cycles for 10^7 copies of *wzy*.

The specificities of the PCR assays targeting the *E. coli* O45

TABLE 3. PCR conditions used for the different assays

PCR gene(s)	Primer or primer pair	Temp (°C) [(time in s)]		Amplicon size(s) (bp)
		Annealing	Extension	
Singleplex PCR				
O45 *wzx*	O45*wzx*1, O45*wzx*2	59 (0)	72 (30)	527, 255
O45 *wzy*	O45*wzy*1, O45*wzy*2	59 (0)	72 (30)	497, 451
O55 *wzx*	O55*wzx*1, O55*wzx*2	50 (0)	72 (8)	150, 683
O55 *wzy*	O55*wzy*1	50 (0)	72 (8)	147
Multiplex PCR				
O45 (*wzx* and *wzy*)	O45*wzx*2, O45*wzy*2	59 (0)	72 (30)	255, 451 (Fig. 1A)
O55 (*wzx* and *wzy*)	O55*wzx*2, O55*wzy*1	55 (0)	72 (32)	683, 147 (Fig. 1B)
O45 (*wzx*) and O55 (*wzx*)	O45*wzx*2, O55*wzx*2	55 (0)	72 (32)	255, 683 (not shown)

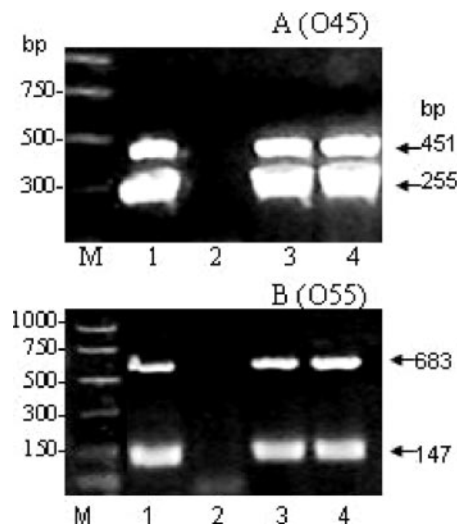

FIG. 1. Multiplex PCRs targeting the *E. coli* O45 *wzx* and *wzy* (A) and O55 *wzx* and *wzy* (B) genes. (A) Lane M, molecular weight markers; lane 1, *E. coli* O45 (positive control) (multiplex PCR showing the amplified *wzx* [255 bp] and *wzy* [451 bp] genes); lane 2, *E. coli* K-12 (negative control); lane 3, results from a chicken fecal sample spiked with *E. coli* O45 at 10^6 CFU/0.2 g; lane 4, result from a chicken fecal sample spiked with *E. coli* O45 at 10^8 CFU/0.2 g. (B) Lane M, molecular weight markers; lane 1, *E. coli* O55 (positive control) (multiplex PCR showing the amplified *wzx* [683 bp] and *wzy* [147 bp] genes); lane 2, *E. coli* K-12, (negative control); lane 3, results from a chicken fecal sample spiked with *E. coli* O55 at 10^6 CFU/0.2 g; lane 4, results from a chicken fecal sample spiked with *E. coli* O55 at 10^8 CFU/0.2 g.

and O55 *wzx* and *wzy* genes were determined by using 168 O standard reference strains. Only *E. coli* O45 and O55 standard reference strains were positive for the O45 *wzx* and *wzy* and O55 *wzx* and *wzy* genes, respectively. Fifty-seven strains belonging to serogroup O45 collected from different sources over the last twenty years exhibited the presence of *wzx* and *wzy* by the PCR. Forty-seven randomly selected *E. coli* isolates belonging to serogroups other than O45 were negative for the presence of the *E. coli* O45 *wzx* and *wzy* genes. On the other hand, when 119 isolates belonging to *E. coli* serogroup O55 selected from our reference collection isolated during the past thirty-seven years were tested for the presence of the O55 *wzx* and *wzy* genes, 17 isolates out of 119 (14.2%) were negative for both genes. The 17 isolates that did not exhibit the presence of O55 *wzx* and *wzy* were again serotyped by conventional agglutination reactions using 179 different O antisera raised in rabbits against the standard reference strains as described previously (11). Out of the 17 isolates that were previously designated O55, 9 cross-reacted with antisera raised against *E. coli* O83, O55, and O22, 4 cross-reacted with O55 and O83, 2 cross-reacted with O55 and O23, and 2 showed a very weak reaction with the O55 antiserum, indicating that these strains may not be O55 but could be related to some other O-antigenic types (Table 4). It is not uncommon to find cultures that cross-react with O22, O83, O55, and O23 antisera. In our large collection of *E. coli* strains, there were 29 isolates that cross-reacted with O83 and O22, 8 cultures that cross-reacted with O55 and O83, 5 cultures that cross-reacted with O22 and O55, and 3 strains that cross-reacted with O23 and O55 antisera. Therefore, these 17 isolates were probably not O55 and cross-reacted with the O55 antiserum. Long PCR performed with all of these isolates, targeting the regions between *gnd* and JUMP-start, exhibited product sizes that were different than that of the reference O55 strain. EcoRI was used to digest the amplified gene cluster of all these isolates, and restriction fragment length polymorphism (RFLP) profiles were determined. While the RFLP pattern for O55 standard strain exhibited five fragments of the expected sizes, none of the other isolates showed a profile similar to the standard (data not shown). It was apparent that these 17 isolates did not belong to serogroup O55. Therefore, the PCR assays were more specific for *E. coli* serogroup O55 strains than conventional serotyping. None of the 21 non-*E. coli* bacteria exhibited the presence of the O45 and O55 *wzx* or *wzy* genes by the PCR exhibiting the specificity of the reactions.

We have developed conventional (singleplex), multiplex, and real-time PCR assays for detection and typing of *E. coli* serogroups O45 and O55. The assays were found to be highly specific for the respective serogroups and can potentially replace conventional serotyping assays that are time-consuming and less specific. The PCR assays were used to detect *E. coli* O45 and O55 serogroup strains in spiked fecal samples and can potentially be used for detecting the presence of these serogroups in food, fecal, and environmental samples.

Nucleotide sequence accession number. The DNA sequence of the 14,483-bp region containing the O45 O-antigen gene cluster has been assigned GenBank accession no. AY771223.

TABLE 4. Specificities of 119 putative *E. coli* O55 isolates and 68 non-O55 *E. coli* isolates and other bacteria

Serological reaction	No. of cultures	Sources (no.)[b]	Conclusions from RFLP analyses of O-antigen gene cluster	No. of positive isolates	
				O55 *wzx* PCR	O55 *wzy* PCR
O55	102	Humans, animals, and environment	Not tested	97	98
O55, O83	4	Cows (3) and unlisted (1)	Not O55	0	0
O55, O83, O22	9	Humans (7), mouse (1), and unlisted (1)	Not O55	0	0
O55, O23	2	Chicken (1) and environment (1)	Not O55	0	0
O55 (weak reaction)[a]	2	Sea gull (2)	Not O55	0	0
Non-O55	47	Human, animals, and environment	Not tested	0	0
Non-*E. coli*	21	ATCC and unknown	Not tested	0	0

[a] These strains may not be O55 but may be an unidentified O type and/or related to O55.
[b] ATCC, American Type Culture Collection.

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