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Detection of O antigens in *Escherichia coli*

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Abstract

Lipopolysaccharide on the surface of *Escherichia coli* constitutes the O antigens which are important virulence factors that are targets of both the innate and adaptive immune systems and play a major role in host–pathogen interactions. O antigens are responsible for antigenic specificity of the strain and determine the O serogroup. The designation of O serogroups is important for classifying *E. coli* strains, for epidemiological studies, in tracing the source of outbreaks of gastrointestinal or other illness, and for linking the source to the infection. For conventional serogroup identification, serotyping by agglutination reactions against antisera developed for each of the O serogroups has been used. In the last decade, many O-antigen gene clusters that encode for the enzymes responsible for the synthesis of the variable oligosaccharide region on the surface of the bacteria have been sequenced and characterized. Unique gene sequences within the O-antigen gene clusters have been targeted for identification and detection of many O groups using the polymerase chain reaction and microarrays. This review summarizes current knowledge on the DNA sequences of the O-antigen gene clusters, genetic-based methods for O-group determination and detection of pathogenic *E. coli* based on O-antigen and virulence gene detection, and provides perspectives on future developments in the field.

Keywords: lipopolysaccharide, serotyping, agglutination, sequencing, RFLP, PCR, virulence, microarrays

Introduction

Escherichia coli is one of the most widely studied organisms in the microbial world. Non-pathogenic strains of *E. coli* are part of the normal flora of the gastrointestinal tract and for many years have been used in the laboratory as a host organism for recombinant DNA techniques; pathogenic strains have been implicated in causing a wide range of intestinal and extra-intestinal diseases in humans and animals (Kaper and Nataro, 2004). The species *E. coli*, belonging to the family Enterobacteriaceae, is a Gram-negative flagellated rod-shaped bacterium. There is enormous diversity within the species, which is differentiated into subgroups based on many different physiological, morphological and antigenic characteristics.

Initially, *E. coli* isolates were differentiated from one another by biotypes that were based on fermentation or non-fermentation of a range of carbohydrate substrates. It was soon realized that this method was not optimal for distinguishing strains in epidemiological investigations as it was limited in its discriminatory power. Therefore, immunogenicity of the bacterial surface structures was considered as a potentially more effective method. The combination of three principal surface antigens (O antigens that are a part of the lipopolysaccharide (LPS), the flagellar H antigens and the K capsular antigens (O:K:H)) was developed as a method for subtyping *E. coli* strains. However, only a few laboratories had the ability to type the K antigen, and therefore, serotyping based on the O and H antigens became the

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'gold standard' for *E. coli*. The responsibility for maintaining the reagents for an international typing scheme and for aspects of research in this field rests with the WHO International *Escherichia* and *Klebsiella* Centre in Copenhagen, but scientists all over the world contribute to new knowledge in this area.

Further refinement by combining serotypes with biotypes based on phage types, virulence markers, phylogenetic profile and genotypic characteristics has now been adopted for distinguishing *E. coli* strains. Within a serogroup several subgroups may be identified by the presence of several H antigens associated with the O antigen. The O:H combination is referred to as a serotype. Within a serotype there may be numerous subtypes that are often identified by a variety of gene-based methods.

In the 1940s, Kaufmann (1943, 1944, 1947) attempted to classify *E. coli* by serological methods, and by 1945, he successfully classified *E. coli* into 20 O groups on the basis of the antigenic scheme he developed by using boiled cultures of *E. coli* for O antisera production. The antisera generated against the O groups were tested with boiled cultures of unknown strains for agglutination reactions for identification of the 20 O groups. Five additional O groups were subsequently added (Knipschildt, 1945). Ørskov *et al.* (1977) presented a comprehensive serotyping scheme comprising 164 O groups, which has been the basis for O classification for taxonomic and epidemiological studies and for distinguishing strains during outbreaks and for surveillance purposes. Later, Ørskov *et al.* (1984) added 6 O groups, O165 through O170, based in part on a selection from 8 O groups that had previously been called OX1 to OX8 by Ewing *et al.* (1956); OX1 was designated as O170, OX2 as O169, OX8 as O166 and OX5 as O168. OX4 and OX6 were found to be similar to O146 and O171, respectively (Ewing, 1972; Scheutz *et al.*, 2004), and OX3 and OX7 were never established as serotypes until 2004 when Scheutz *et al.* (2004) designated OX3 and OX7 as O174 and O175. The nucleotide sequence of the OX6 O-antigen gene cluster has been found to be similar to that of O45 (Fratamico *et al.*, unpublished data). Ørskov *et al.* (1991) reported two more serogroups, O172 and O173, and Scheutz *et al.* (2004) added O176 to O181. Currently there are 174 O groups, identified as O1–O181 O groups except for O groups O31, O47, O67, O72, O93, O94 and O122 which were determined to be no longer valid O groups.

It should be emphasized that the international typing scheme is limited to the typing of *E. coli* which appear to be important, primarily for their involvement or potential involvement in disease. There are large numbers of strains of *E. coli* that are designated as 'untypeable' because they do not react with antisera in the established typing scheme. These untypeable strains include pathogenic ones (Duda *et al.*, 2011). Some of these strains may be assigned to an O serogroup, based on genetic methods (DebRoy *et al.*, 2010).

LPS

The cell envelope of Gram-negative bacteria is composed of the outer and inner membranes that are separated by the periplasm. The outer membrane is composed of phospholipids, LPSs, membrane proteins and lipoproteins (Bos *et al.*, 2007). LPS, also known as endotoxin, consists of three components: the hydrophobic membrane anchor lipid A region, which is associated with the toxicity of the LPS and is well conserved among Gram-negative bacteria (Raetz and Whitfield, 2002), the distal O-antigen polysaccharide region that is exposed to the surface and the core polysaccharide region that connects the two.

The lipid A moiety consists of a β-1,6-linked D-glucosamine disaccharide carrying ester- and amide-linked 3-hydroxy fatty acids at the 2, 3, 2′ and 3′ positions and phosphate groups at the 1, 4′ positions. The lipid A biosynthetic pathway has been characterized (Raetz and Whitfield, 2002; Wang and Quinn, 2010). The first step in the biosynthesis of LPS in *E. coli* is catalyzed by UDP-*N*-acetylglucosamine (UDP-GlcNAc) acyltransferase (LpxA) leading to the reversible transfer of the R-3-hydroxyacyl chain from R-3-hydroxyacyl acyl carrier protein to the glucosamine 3-OH group of UDP-GlcNAc. A number of enzymes sequentially convert this structure into disaccharide-1-P, Kdo_2-lipid A, core-lipid A and eventually LPS (Wang and Quinn, 2010). The lipid A structure is more conserved than that of the outer core oligosaccharide region, which shows more variability among bacterial species. There are five types of core in *E. coli*, named R1–R4 and K-12. The core oligosaccharides are sequentially assembled on lipid A at the cytoplasmic surface of the inner membrane. This involves a number of glycotransferases that are membrane bound and use nucleotide sugars as donors. Core oligosaccharides can be divided into two regions, inner and outer core. The inner oligosaccharide component of the core connects to lipid A and the outer component connects to the O antigen repeating units (Raetz *et al.* 2007).

LPS is responsible for stimulation of the innate immune system. The response from the host depends on the particular structure of lipid A, which is the bioactive component of LPS and is responsible for the toxic effect of Gram-negative bacterial infections (Galanos *et al.*, 1985). LPS is recognized by the Toll-like receptor 4 (TLR4) present on the surface of monocytes, macrophages, neutrophils and dendritic cells, cells of the innate immune system (Poltorak *et al.*, 1998; Akira *et al.*, 2006). The lipid A of *E. coli*, composed of two phosphate groups and six acyl chains containing 12 or 14 carbons, is a powerful activator of the innate immune system (Golenbock *et al.*, 1991).

The O antigens are thermostable and are found in all smooth bacteria within the Enterobacteriaceae, which means that the bacteria retain their immunogenicity, agglutinating and agglutinin-binding capacity at boiling

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temperature. This property has been exploited for serological identification of O antigens where the bacteria are boiled to react with antisera for agglutination. Bacteria that have mutated and lost the O antigen specificity are referred to as 'rough' forms and cannot be serotyped by agglutination reactions as they have lost the ability to synthesize the O antigens. Interestingly, although LPS contributes to virulence, rough strains of *E. coli* have been recognized as highly virulent. For example, O rough:H7 Shiga toxin-producing *E. coli* (STEC) implicated in bloody diarrhea have been shown to be O157:H7 organisms that failed to express their O antigen, because of an insertion in one of the genes in the O-antigen gene cluster (Rump *et al.*, 2010a, b).

Biosynthesis of the O antigens

The genes in the *E. coli* O-antigen gene clusters encode for enzymes responsible for O antigen biosynthesis. There are three major gene classes: nucleotide sugar synthesis genes, sugar transferase genes and O unit processing genes. These genes are responsible for carrying out three distinct processes that are involved in the synthesis and translocation of the O antigen (Bronner *et al.*, 1994; Reeves *et al.*, 1996; Keenleyside and Whitfield, 1996; Daniels *et al.*, 1998; Linton and Higgins, 1998; Samuel and Reeves, 2003; Liu *et al.*, 2008; Wang *et al.*, 2010). Nucleotide sugar synthesis genes encode for proteins that are responsible for sugar synthesis specific to certain O groups. Enzymes of the second group, the glycosyl transferase proteins, transfer various precursor sugars for the specific linkages in the O antigen to form oligosaccharides on the carrier lipid undecaprenyl phosphate (UndP), situated in the inner membrane facing the cytoplasm (Reeves, 1994). The O antigen processing proteins, the Wzx (O antigen flippase) and Wzy (O antigen polymerase), are involved in translocation of the O units across the membrane and in polymerization. The O unit is synthesized by sequential transfer of sugars and other constituents to the first sugar, which is then translocated and flipped across the membrane by the protein Wzx to the periplasmic face. These are further polymerized by Wzy (Mulford and Osborn, 1983; McGrath and Osborn, 1991; Reeves and Wang, 2002; Liu *et al.*, 2008). The number of O units in the final O antigen is regulated by Wzz. The Wzx and Wzy proteins are both hydrophobic and have about 12 predicted transmembrane regions, and a long cytoplasmic region is predicted between the transmembrane domains in Wzy. In some of the *E. coli* O groups such as O8, O9, O9a, O52 and O99, the translocation of the O antigen is carried out by an ABC transporter protein, Wzm, which is responsible for the export, and Wzt, the ATP-binding component.

O antigens are composed of repeat units of oligosaccharides (O unit) comprising sugar residues, which vary considerably and differ in their arrangement and linkage between and within the O units, making the O antigen the most variable region of the bacterial cell. The O antigens are highly immunogenic and variability among the groups provides the basis for serotyping and classification of *E. coli*. Carbohydrate analysis methods have been valuable in the identification of the structures of numerous O antigens (MacLean *et al.*, 2006, 2010; Chafchaouni-Moussaoui *et al.*, 2011).

O antigens may contribute to virulence of the organism, and certain O groups are associated with high virulence. It has been shown that specific clones within certain serotypes are associated with specific diseases (Ørskov *et al.*, 1976; Achtman and Pluschke, 1986). *E. coli* O18:K1 was shown to cause newborn meningitis and neonatal bacteremia, multiplying in the bloodstream of infected newborn rats; this pathogen was resistant to the bactericidal effects of complement in the absence of specific antibodies. Loss of the O antigen resulted in the strain becoming more sensitive to the bactericidal effects of the classical complement pathway (Pluschke *et al.*, 1983). Differences in virulence among certain serotypes of *E. coli* were dependent on specific cell surface structures such as O antigens (O1, O7, O16, O18 and O75), capsule and fimbriae (Kusecek *et al.*, 1984). Outbreaks of disease due to *E. coli* O157:H7, causing severe gastrointestinal illness and affecting 47 people, were described for the first time in 1983 (Riley *et al.*, 1983; Remis *et al.*, 1984). Since then, many reports have established *E. coli* O157:H7 carrying Shiga toxin genes as a major pathogen that causes hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Krishnan *et al.*, 1987; Neill *et al.*, 1987). STEC strains belonging to specific O serogroups, including O26, O45, O103, O111, O121 and O145, have been linked to outbreaks of disease that are similar to those caused by O157, but occur at a lower frequency and tend to be less severe (Karmali *et al.*, 2003; Gyles, 2007).

There is evidence to suggest that the O antigens in Enterobacteriaceae play an important role in host–pathogen interactions (West *et al.*, 2005); therefore, it is possible that the presence of specific O antigens may be a factor contributing to disease caused by certain serogroups. In *Yersinia* serotype O8, pathogenesis was examined by analysis of mutants with no O antigen, LPS with one O unit and LPS with random distribution of the O antigen. It was found that LPS mutants with attenuated O antigens were severely impaired in their ability to adhere and could not colonize in the spleen and liver in contrast to the wild type (Bengoechea *et al.*, 2004). Recently, Vigil *et al.* (2011) reported by whole genome screening approaches, including transcriptomic, proteomic, and signature-tagged mutagenesis, that uropathogenic *E. coli* strains in humans expressed or required genes for capsule, LPS, translational machinery, type 1 fimbriae and iron acquisition systems to cause urinary tract infection (UTI). Among extra-intestinal pathogenic *E. coli* (ExPEC) strains that cause UTI, strains belonging to

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serogroups O4 and O6 have been implicated in causing fatal pneumonia in dogs, cats and tigers (Handt *et al.*, 2003; Sura *et al.*, 2007; Carvallo *et al.,* 2010).

Horizontal transfer and recombination can convert *E. coli* from one O-antigen to another. This is well documented in the case of evolution of *E. coli* O157:H7 whose O antigen was changed from O55 to O157 (Tarr *et al.*, 2000). Horizontal transfer by bacteriophage (phage conversion) is well known to cause changes in LPS in *Salmonella*.

O antigen detection

Serotyping

Conventional serotyping for O group identification is based on agglutination reactions, which remains one of the most comprehensive and simple methods for testing O groups. Serotyping is carried out in tubes or 96-well plates or on slides using antisera that are generated by immunization of rabbits with different O group reference strains (Ørskov *et al.*, 1977; Ørskov and Ørskov, 1984). When antisera containing the antibodies for each O group are allowed to react with an unknown O antigen, released by heating the bacteria for 2 h at 100°C, agglutination, or clumping of the bacteria is observed when the O antigen reacts with the specific antiserum. However, if the organism is capsulated or rough and does not carry LPS, the agglutination reaction fails. Sometimes there may be cross reactions with other O groups, resulting in equivocal results.

Slide agglutination is conducted by adding a small amount of culture growing on an agar plate to a droplet of diluted serum on a microscope slide. The slide is rocked for a few seconds, and agglutination is observed by the naked eye. However, the method is not confirmatory and can result in equivocal results. Currently, serotyping is generally performed by observing for agglutination in 96-well plates. The diluted antisera (20 µl) are then dispensed in 96-well microtiter plates, with each well containing antiserum against a different O group. For O serotyping, bacteria are grown overnight to a concentration of 10^8–10^9 colony-forming units (CFU)/ml. The cultures are heated for 2 h at 100°C, diluted with formalinized saline to a McFarland density of 2, and 180 µl of bacterial suspension are added to the antisera in the wells and mixed. The 96-well plates are incubated at 50°C overnight, then checked for agglutination reactions. Titers, which indicate the relative strength of the antiserum, are expressed as the reciprocal of the highest dilution that resulted in agglutination. A comparison of the titers of the antisera of known potency against the unknown *E. coli* isolate and the homologous strain is a rough guide to the relationship of the unknown O antigen to that of the O antigen used to prepare the reference antiserum.

Restriction fragment length polymorphism (RFLP) analysis of O-antigen gene clusters

O-antigen gene clusters are located at position 44–45 min on the chromosome of *E. coli*, at 200–2000 base pairs (bp) upstream of *gnd* encoding for 6-phosphogluconate dehydrogenase (Batchelor *et al.*, 1992; Bastin *et al.* 1993). There is a highly conserved 39-bp JUMPstart sequence upstream of the O-antigen gene cluster (Hobbs and Reeves, 1994). Using primer sequences complementary to these regions, Wang and Reeves (1998) sequenced the *E. coli* O157 O-antigen gene cluster and determined specific sequences that could be used to identify the O157 serogroup. Subsequently, the genes for many O groups have been sequenced using this strategy. Coimbra *et al.* (2000) used the same strategy and amplified the O-antigen gene clusters of 148 O serogroups and digested the amplification product with the restriction enzyme *Mbo*II. Profiles were compared and 147 patterns were obtained. Although the authors claimed that the patterns were unique, 13 RFLP patterns were shared by two or more O groups, making it difficult to substitute this method for serotyping for routine diagnostic testing.

PCR assays for O antigen detection

The O-antigen gene clusters for many O groups have now been sequenced (Marolda and Valvano, 1993; Bastin and Reeves, 1995; Amor and Whitfield, 1997; Wang and Reeves, 1998; Wang L *et al.*, 1998, 2001, 2002, 2007, 2009, 2010, Paton and Paton, 1999; Shepherd *et al.*, 2000; D'Souza *et al.*, 2002, 2005; Grozdanov *et al.*, 2002; Perelle, *et al.*, 2002; Shao *et al.*, 2003; Fratamico *et al.*, 2003, 2005, 2009a, b, 2010; Feng *et al.*, 2004a, b, 2005a, b, 2007; Guo *et al.*, 2004, 2005; Beutin *et al.*, 2005a, b, 2007; DebRoy *et al.*, 2005; Cheng *et al.*, 2006, 2007; Cunneen and Reeves, 2007; Han *et al.*, 2007; Liu Y *et al.*, 2007, 2008; Liu B *et al.*, 2010; Ren *et al.*, 2008; Perepelov *et al.*, 2009, 2011a, b; Wang Q *et al.*, 2009, 2010a, b; Li *et al.*, 2010a, b). DNA sequences for about 95 O-antigen gene clusters have been deposited in GenBank so far, and the accession numbers are listed in Table 1. The genetics of the O-antigen gene clusters are directly correlated with the structural variations of the O antigens. Genes in the O-antigen gene clusters for all O groups sequenced are transcribed in one direction (Fig. 1).

Nucleotide sequences of the O-antigen gene clusters for O107 and O117 are homologous (Wang *et al.*, 2009), and these two O groups have been found to cross-react in serotyping assays. More recently, Wang *et al.* (2009) have shown these two O-antigen gene clusters, having 98.6% DNA identity, encode genes that result in differences in 1 sugar residue. They identified a nucleotide substitution in 1 gene which resulted in a Glc-transferase in O117 and a Glc-NAc transferase in O107. The O-antigen gene

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Table 1. Accession numbers in GenBank for all O antigen gene clusters that have been sequenced

O type	Base pairs	ORFs	GenBank No.	O type	Base pairs	ORFs	GenBank No.
2	14,063	15	EU549863	106	10,484	6	DQ000315.1
3	14,049	10	EU694097	107	10,884	11	EU694095
4	14,531	13	AY568960	109	16,597	14	HM485572
6	11,312	8	AJ426045	111	14,516	11	AF078736
7	15,943	14	AF125322	112ab	10,812	8	EU296413
8	6909	6	AF013583	112ac	8392	8	EU296405
13	12,369	8	EU296422	113	14,263	8	AF172324
15	11,893	9	AY647261	114	13,272	12	AY573377
17	13,360	10	AF285971.1	115	15,588	14	GU068041
18	12,075	10	GU299793	117	10,883	11	EU694096
21	8041	6	EU694098	118	13,283	12	DQ990684
22	8132	8	DQ851855	119	17,610	16	GQ499368
24	18,772	9	DQ220292	121	15,155	14	AY208937
25	17,547	14	GU014554	123	15,783	15	DQ676934
26	13,270	12	AF529080	124	10,311	9	EU296419
27	9510	7	GU014555	126	11,783	10	DQ465248
28ac	8579	7	DQ462205	127	13,663	12	AY493508
29	14,249	12	EU294173	128	19,013	13	AY217096
32	7286	7	EU296410	129	13,480	8	EU296424
35	14,060	12	FJ940774	130	10,990	8	EU296421
40	13,698	6	EU296417	135	11,364	8	EU296423
45	14,483	13	AY771223	137	9205	10	GU068043
52	18,900	16	AY528413	138	14,139	11	DQ109551
53	10,355	10	EU289392	139	12,507	10	DQ109552
55	27,730	14	AF461121	141	15,601	12	DQ868765
56	13,075	9	DQ220293	143	14,105	10	EU294164
58	17,770	13	EU294175	145	16,932	15	AY647260
59	16,573	12	AY654590	146	11,888	11	DQ465249
61	15,464	14	GU220362	147	10,319	8	DQ868766
63	13,492	12	EU549862	148	10,241	9	DQ167407
66	15,137	13	DQ069297	149	11,064	9	DQ091854
71	13,743	12	GU445927	150	13,552	11	EU294168
73	10,486	6	DQ000313.1	151	13,283	12	DQ990685
75	12,945	11	GU299795	152	11,575	10	EU294170
77	10,485	6	DQ000314.1	155	12,755	9	AY657020
78	12,655	12	FJ940775	157	14,002	12	AF061251
79	12,892	13	EU294162	158	14,597	11	GU068044
85	11,203	8	GU299798	159	13,749	11	EU294176
86	14,156	12	AY670704	161	15,878	12	GU220361
87	7267	7	EU294177	164	14,006	8	EU296420
91	10,196	11	AY035396	165	11,292	10	GU068045
98	14,333	11	DQ180602	167	12,864	9	EU296408
99	17,685	12	FJ940773	168	14,804	12	EU296403
103	12,033	12	AY532664	172	12,850	10	AY545992
104	12,105	10	AF361371	173	13,697	11	GU068046
105	11,106	11	EU294171	174	5660	5	DQ008592
				177	13,198	13	DQ008593

clusters of *E. coli* O129 and O135 also exhibit 100% homology (Liu Y *et al.*, 2008), and the DNA sequences of O118 and O151 are alike except for two nucleotides that are substituted in O151 thereby changing two amino acids in the proteins that are transcribed (Liu Y *et al.*, 2008). O groups with homologous sequences may be merged to avoid confusion.

Plainvert *et al.* (2007) identified a meningitis-causing extraintestinal pathogenic *E. coli* clone that belonged to serogroup O45; however, there were clear differences in the sequence of the O-antigen gene cluster of this clone (S88) with that of the O45:H2 96-3285 strain that had been previously published (DebRoy *et al.*, 2005). The putative Wzx of strain S88 showed 23% identity and 42% similarity to the putative Wzx of *E. coli* 96-3285. It was suggested that multiple recombination events led to the horizontal acquisition of the new O-antigen gene cluster, referred to as the $O45_{S88}$ antigen, leading to the emergence of a virulent O45:H7 clone.

The O-antigen gene clusters are generally highly heterologous with considerable nucleotide differences between the clusters. However, the predominant genes in the clusters are those involved in nucleotide sugar synthesis, and encoding for synthetases, transferases,

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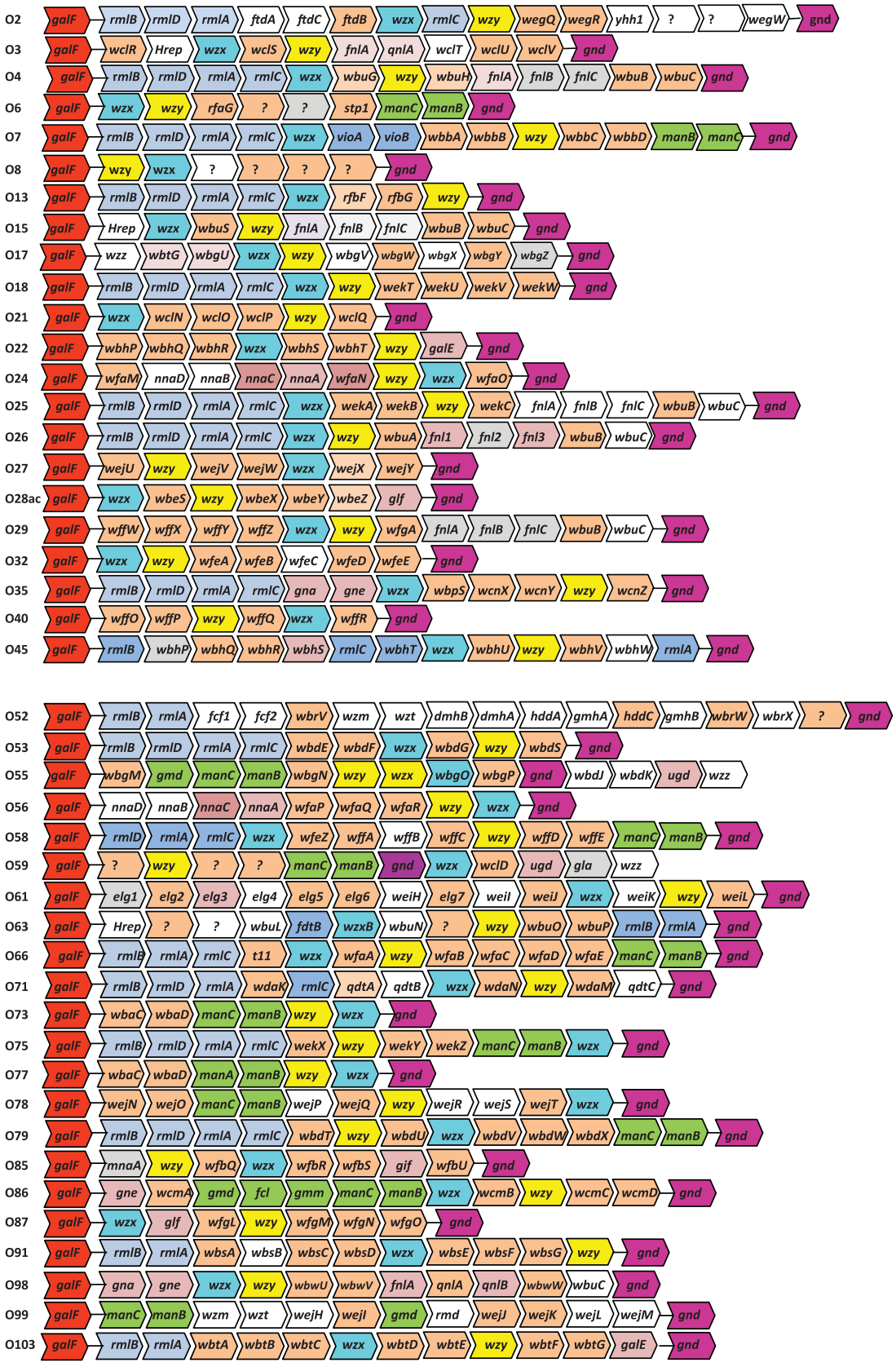

Fig. 1. (Continued)

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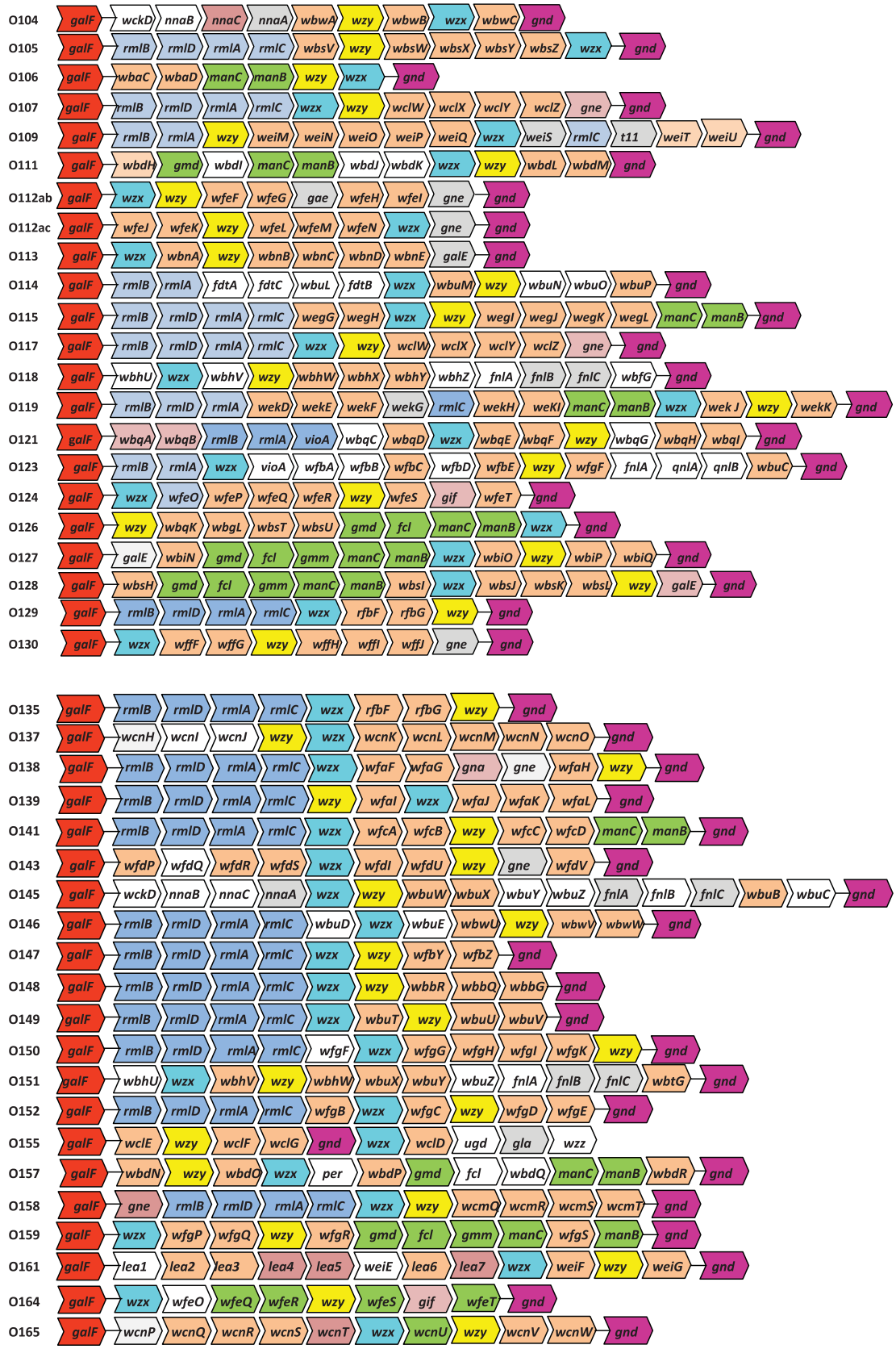

Fig. 1. (Continued)

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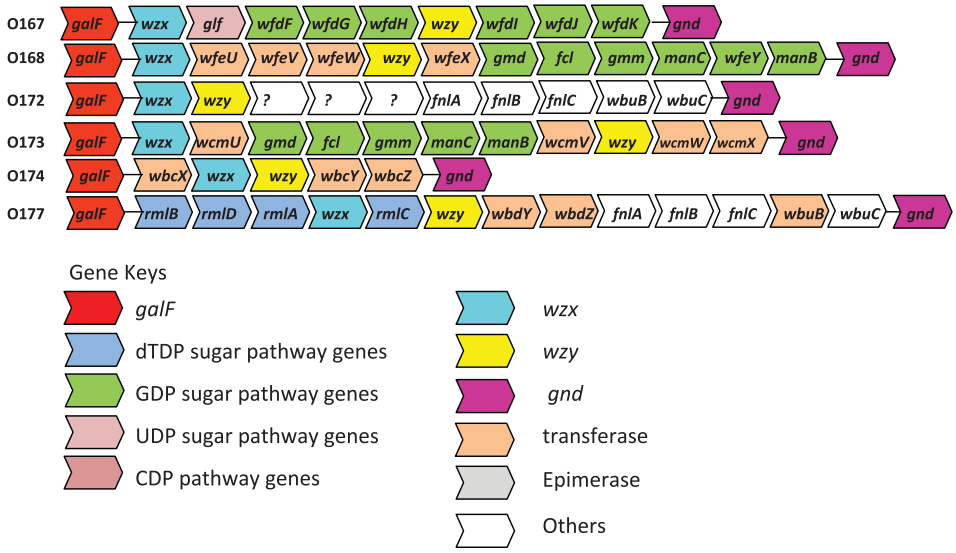

Fig. 1. O antigen gene clusters of all *E. coli* O antigens from GenBank. The arrows represent the location and direction of translation for putative genes in the clusters. The genes are not represented in scale.

epimerases and other enzymes. Although the genes encoding specific proteins may have similarities in functions among the O groups, the DNA sequences may be quite variable. In most of the clusters, two genes, encoding the O antigen flippase (*wzx*) and the O antigen polymerase (*wzy*) responsible for O antigen translocation across the membrane and for polymerization, are unique, and therefore have been used as targets in genetic-based methods for serogroup identification (Fig. 2). A list of primer sequences used for detecting the O groups is presented in Table 2. Singleplex, multiplex and real-time PCR assays that are highly sensitive and specific have been developed for the detection and identification of many O groups of *E. coli* (Bastin and Reeves, 1995; Wang *et al.*, 1998, 2001, 2002, 2005, 2009, 2010a, b; Grozdanov *et al.*, 2002; Perelle *et al.*, 2002; Shao *et al.*, 2003; Fratamico *et al.*, 2003, 2005, 2009a, b, 2010, 2011; DebRoy *et al.*, 2004, 2005, 2010, 2011; Feng *et al.*, 2004a, b, 2005a, b, 2007; Guo *et al.*, 2004, 2005; Beutin *et al.*, 2005a, b, 2007; Cheng *et al.*, 2007; Liu Y *et al.*, 2007, 2008; Ren *et al.*, 2008; Han *et al.*, 2007; Goswami *et al.*, 2010; Li *et al.*, 2006, 2010a, b).

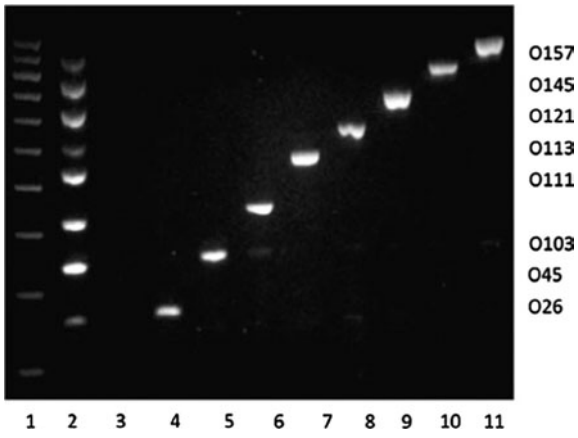

Fig. 2. Multiplex polymerase chain reaction (m-PCR) assay for detecting STEC O-groups targeting the *wzx* gene. Lane 1, molecular weight markers; lane 2, pooled amplified DNA generated from eight STEC O-groups in one m-PCR reaction; lane 3, negative control strain *E. coli* K12; lane 4, O26 (155 bp); lane 5, O45 (238 bp); lane 6, O103 (321 bp); lane 7, O111 (438 bp); lane 8, O113 (514 bp); lane 9, O121 (628 bp); lane 10, O145 (750 bp); lane 11, O157 (894 bp). Reproduced with permission from DebRoy *et al.* (2011).

Detection of E. coli serogroups belonging to different pathotypes

Use of multiplex PCR assays targeting a serogroup-specific region within the *E. coli* O antigen gene cluster, for example, the *wzx* or *wzy* gene, as well as genes encoding for the Shiga toxins or other virulence genes has been reported (Fratamico *et al.*, 2005, 2009a, b, 2010). The multiplex assays can be used to simultaneously identify the *E. coli* serogroup and determine the specific pathotype, such as STEC (Fig. 3) or enteroinvasive *E. coli* (EIEC). Alternatively, food samples or other types of samples can be subjected first to screening for virulence genes of interest, followed by testing for specific *E. coli* O groups of interest, targeting genes within the O-antigen gene cluster (Perelle *et al.*, 2004; Fratamico *et al.*, 2011). Bugarel *et al.* (2010) utilized GeneDisc® array (Gene Systems, Bruz, France) technology to identify 12 *E. coli* O serogroups and 7 H flagellar types, as well as the presence of virulence genes in one platform.

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Table 2. Primer sequences for the detection of O groups by PCR

O group	Gene	Primer sequence (5′–3′)	Amplicon (bp)	Reference
O1	*wzx*-F	GTGAGCAAAAGTGAAATAAGGAACG	1098	Li D *et al.* (2010a)
	wzx-R	CGCTGATACGAATACCATCCTAC		
O2	*wzx*-F	TGGCCTTGTTCGATATACTGCGGA	819	Fratamico *et al.* (2010)
	wzx-R	TCACGAGCTGAGCGAAACTGTTCA		
	wzy-F	TGCAACTCATTGGTCTGCTTTGCC	351	Fratamico *et al.* (2010)
	wzy-R	CGGAAAGCCATAACAGGTAGAGAG		
O3	*wzy*-F	ACTCCGATACAGAACG	549	Ren *et al.* (2008)
	wzy-R	AAGTGGGATTATCATAT		
O4	*wzx*-F	TTGTTGCGATAATGTGCATGTTCC	664	Li D *et al.* (2010a)
	wzx-R	AATAATTTGCTATACCCACACCCTC		
O6	*wzy*-F	GGATGACGATGTGATTTTGGCTAAC	783	Li D *et al.* (2010a)
	wzy-R	TCTGGGTTTGCTGTGTATGAGGC		
O7	*wzx*-F	CTATCAAAATACCTCTGCTGGAATC	610	Li D *et al.* (2010a)
	wzx-R	TGGCTTCGAGATTAAACCTATTCCT		
O8	orf469	CCAGAGGCATAATCAGAAATAACAG	448	Li D *et al.* (2010a)
	orf469	GCAGAGTTAGTCAACAAAAGGTCAG		
O9	*wzt*-F	TGGGTGTTAAAAGACATCAA	1142	Liu B *et al.* (2010)
	wzt-R	CCCAGAAATCCATGCTC		
O15	*wzy*-F	TCTTGTTAGAGTCATTGGTGTATCG	183	Li D *et al.* (2010a)
	wzy-R	ATAAAACGAGCAAGCACCACACC		
O16	*wzx*-F	GGTTTCAATCTCACAGCAACTCAG	302	Li D *et al.* (2010a)
	wzx-R	GTTAGAGGGATAATAGCCAAGCGG		
O18	*wzx*-F	GTTCGGTGGTTGGATTACAGTTAG	551	Li D *et al.* (2010a)
	wzx-R	CTACTATCATCCTCACTGACCACG		
O21	*wzx*-F	CTGCTGATGTCGCTATTATTGCTG	209	Li D *et al.* (2010a)
	wzx-R	TGAAAAAAAGGGAAACAGAAGAGCC		
O22	*wzx*-F	TGTCGCCACTACTTTCCGCGTTTA	458	Fratamico *et al.* (2009a)
	wzx-R	AGCCCATGACATTACTACGGCACT		
O24	*wzx*-F	TGATTGTTTGGTAAGAC	345	Cheng *et al.* (2006)
	wzx-R	AGCCATGCTTTTA		
	wzy-F	TGTTGGTCCTAGGGTA	317	Cheng *et al.* (2006)
	wzy-R	TCAGAGTTCAAAATG		
O25	*wzy*-F	AGAGATCCGTCTTTTATTTGTTCGC	230	Li D *et al.* (2010a)
	wzy-R	GTTCTGGATACCTAACGCAATACCC		
O26	*wzx*-F	GCGCTGCAATTGCTTATGTA	152	DebRoy *et al.* (2004)
	wzx-R	TTTCCCCGCAATTTATTCAG		
	wzy-F	TAAATTGCGGGGAAAGAATG	256	DebRoy *et al.* (2004)
	wzy-R	GACTTCATGGGTACCGCCTA		
O28ac	*wzx*-F	ACCAGAGCAAGGACGATTTGTCA	554	Fratamico *et al.* (2010)
	wzx-R	CAACTTTAACTTTCCCAAGCGCGG		
	wzy-F	GGTAATACACTTGCTGTGGTGGGT	218	Fratamico *et al.* (2010)
	wzy-R	ATGATTGACCATCCCAGGCCGTAT		
O45	*wzx*-F	CCGGGTTTCGATTTGTGAAGGTTG	527	DebRoy *et al.* (2005)
	wzx-R	CACAACAGCCACTACTAGGCAGAA		
	wzy-F	GAAATTATGCCATCTTGGCGAGCG	497	DebRoy *et al.* (2005)
	wzy-R	CATGTGAAGCCTGAAGGCAAACTC		
O52	*wzm*-F	TTTGGTCGCAGCGTTAGT	543	Feng *et al.* (2004a)
	wzm-R	CAACTCGTGGGAAGATGA		
O55	*wzx*-F	AATGGAACATTGCAACAGCA	150	DebRoy *et al.* (2005)
	wzx-R	TGTGGATTCCAGAAAAGCAA		

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Table 2 (Continued)

O group	Gene	Primer sequence (5′–3′)	Amplicon (bp)	Reference
O56	*wzx*-F	TGTCGGTGTTGAAGTCTAT	692	Cheng *et al.* (2006)
	wzx-R	GCTAAAATTAATTTTATT		
	wzy-F	CTTAGGGTGAATGTAGGC	255	Cheng *et al.* (2006)
	wzy-R	TAGTTGATGTTAGAGGGA		
O66	*wzx*-F	TTGCGATGGCAGGAATAA	469	Cheng *et al.* (2007)
	wzx-R	GCTCCAACGGCTAGTGAA		
	wzy-F	CGAGCAAATTAAATCCAC	301	Cheng *et al.* (2007)
	wzy-R	TCAACACTAAACGAAACG		
O71	*wzx*-F	GCATTATTAGCCACTTCAA	344	Hu *et al.* (2010)
	wzx-R	AGCCGTATCATTTAGAGCAGA		
	wzy-F	TGGCTTTATTGGGCTTATA	277	Hu *et al.* (2010)
	wzy-R	CTTAATGGGTCAATCTGCGGGATACCAAAA		
O75	*wzy*-F	GAGATATACATGGGGAGGTAGGCT	511	Li D *et al.* (2010a)
	wzy-R	ACCCGATAATCATATTCTTCCCAAC		
O78	*wzx*-F	GGTATGGGTTTGGTGGTA	992	Liu B *et al.* (2010)
	wzx-R	AGAATCACAACTCTCGGCA		
O83	*wzx*-F	GTACACCAGGCAAACCTCGAAAG	362	Li D *et al.* (2010a)
	wzx-R	TTCTGTAAGCTAATGAATAGGCACC		
O85	*wzx*-F	GTTGTAGATTTCGGGATA	759	Perepelov *et al.* (2011a)
	wzx-R	TTAGCAGCCAGACAAA		
	wzy-F	AGTGGTCATCGCTCAT	352	Perepelov *et al.* (2011a)
	wzy-R	CCTTACTACCTTCGTC		
O86	*wzy*-F	GAGTTATTTTGGTTCACCCTT	731	Liu B *et al.* (2010)
	wzy-R	TAGCCCACCTATGAATAGAGC		
O91	*wzx*-F	TTGCATCTGGCGCAATAAACACGG	616	Fratamico *et al.* (2009a)
	wzx-R	ACACCATCCCAAATACCTGCTTGC		
	wzy-F	CGCATTTAAGGACTGGCTGT	277	Fratamico *et al.* (2009a)
	wzy-R	GTAGCAGATATGCCGACCGT		
O101	*wzm*-F	GTGTTACTTTCATATCGTCCAG	507	Liu B *et al.* (2010)
	wzm-R	ATGCAATGCGGTTTCTAC		
O103	*wzx*-F	TTGGAGCGTTAACTGGACCT	321	Fratamico *et al.* (2005)
	wzx-R	GCTCCCGAGCACGTATAAG		
	wzy-F	ATACAAATGGCGTGGATTGG	280	Fratamico *et al.* (2005)
	wzy-R	GCCAGTAATTGACGTAACTGCTC		
O104	*wzy*-F	CTTCACGAGGTGTCAAGt	1011	Wang L *et al.* (2001)
	wzy-R	CTATCCGATGATCGTGGT		
	wzx-F	CATTCATGACGCTAGAAC	531	Wang L *et al.* (2001)
	wzx-R	TCACATGCACCAGTTAAG		
O111	*wzx-F*	ATGGTATTAACAGTGAAA	1263	Wang L *et al.* (1998)
	wzx-R	ATAGACATTTTTCGCTCT		
O113	*wzx*-F	GGGTTAGATGGAGCGCTATTGAGA	771	DebRoy *et al.* (2004)
	wzx-R	AGGTCACCCTCTGAATTATGGCAG		
	wzy-F	GCATGTATGATGCATAGCTTCGCC	419	DebRoy *et al.* (2004)
	wzy-R	TGATATCGTTCGCTAACCACCCA		
O114	*wzx*-F	CAGGTTTAAGTTGGGTA	603	Feng *et al.* (2004b)
	wzx-R	AAGAAGAAAGTCTGGGTA		
O115	*wzx*-F	TTAGGAATGGCTGGTATGGG	496	Wang Q *et al.* (2010a)
	wzx-R	GCATGGCGAAATAATAAACA		
	wzy-F	CGTCGTGATGTGCATTGTTT	327	Wang Q *et al.* (2010a)
	wzy-R	GCAACACTAAACGCCTCTTT		

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Table 2 (Continued)

O group	Gene	Primer sequence (5′–3′)	Amplicon (bp)	Reference
O117	*wzy*-F	TGTTCTCCACTGCGATCATAGGT	518	Liu Y *et al.* (2007)
	wzy-R	ACATAGAGTACCCGACACCATCAC		
	wzy-F	TGAAATACTCGGTTTCAGCAAGAG	298	Liu Y *et al.* (2007)
	wzy-R	TAGCCAGCAAGGTATGCTGAAGGA		
O118	*wzx*-F	GTG GGA GTC TGA ATC AAG TTG CGA	344	Liu Y *et al.* (2008)
	wzx-R	AGCAACCTTACCCAATCCTAAGGG		
	wzy-F	TGCAAGAGATGGTATTGAGCTGGG	517	Liu Y *et al.* (2008)
	wzy-R	TCCTGAGCCAATTTCTGTAGGTCG		
O119	*wzx*-F	GTTAACAATCAGCTCGATAAAC	650	Liu B. *et al.* (2010)
	wzx-R	TTTGCAAGTAAACACCCTAAAC		
O121	*wzx*-F	AGGCGCTGTTTGGTCTCTTA	310	Fratamico *et al.* (2003)
	wzx-R	TCGCTACCGCTAATGATTCC		
	wzy-F	AGCCGGTAGTGTTGAAAGGA	299	Fratamico *et al.* (2003)
	wzy-R	CTTCAATGAGTGCAGGCAAA		
O123	*wzx*-F	TATCGTCATTATCCACTGTC	668	Beutin *et al.* (2007)
	wzx-R	TTACTATTAGAATAACCGTG		
	wzy-F	GTAGAGGATGGCTTGGGTGG	518	Beutin *et al.* (2007)
	wzy-R	AAATACGGACAGGGTGC		
O126	*wzx*-F	TTAGCTCTCGTAGAGGCTGGTGTT	925	Liu Y *et al.* (2007)
	wzx-R	ATGTCATTCCTGGGACGCGAATGT		
	wzy-F	CGCATTAAATGGACCTGATAAAGCATCG	465	Liu Y *et al.* (2007)
	wzy-R	ACTAGCGCACATATCGTTAGCACG		
O128	*wzx*-F	TCTTGCTTATAGCCAGAATT	1353	Li Y *et al.* (2006)
	wzx-R	AATAAACCGACACCGAAA		
	wzy-F	ATGATTTCTTACGGAGTGC	1035	Li Y *et al.* (2006)
	wzy-R	CTCTAACCTAATCCCTCCC		
O137	*wzx*-F	ATGTTTGGCTAGGGAATGGG	432	Wang Q *et al.* (2010a)
	wzx-R	TCGGATACACGCAAGTCAGT		
	wzy-F	GGGATAGGTTTATTGTTGCA	1107	Wang Q *et al.* (2010a)
	wzy-R	GTTAGCCATCCACCAAGGTA		
O138	*wzx*-F	TTTTATACAGCACCATCAG	460	Wang L *et al.* (2005)
	wzx-R	TAACCATCTAAAGCACAATG		
	wzy-F	TGCCGACAACATTATCAA	507	Wang L *et al.* (2005)
	wzy-R	TCTTTCGGTGGATTTCGT		
O139	*wzx*-F	AGAAACATTACGCCTTGA	507	Wang L *et al.* (2005)
	wzx-R	AGAACAACGGTCAAGAGT		
	wzy-F	GGGATGATTACTGATGCT	261	Wang L *et al.* (2005)
	wzy-R	AACAGCAGCACAAATCAA		
O141	*wzy*-F	TGAACCTGGGTTTACATT	746	Han *et al.* (2007)
	wzy-R	GTACAATTATCATTGCGAGT		
O145	*wzx*-F	ACTGGGATTGGACGTGGATA	222	Fratamico *et al.* (2009b)
	wzx-R	AGGCAAGCTTTGGAAATGAA		
	wzy-F	CTGTTGCTTCAGCCCTTTTC	217	Fratamico *et al.* (2009b)
	wzy-R	GCAGCCCAATATGAAACCAT		
O146	*wzx*-F	AGGGTGACCATCAACACACTTGGA	640	Liu Y *et al.* (2007)
	wzx-R	AGTTCAATACTGTCGCAGCTCCTC		
	wzy-F	ATTCGGGTAACGACCCTGTGTTGA	378	Liu Y *et al.* (2007)
	wzy-R	AGACTGCTAATGCAAGGAACATGG		
O147	*wzy*-F	TGGAAATGCTCTCATTCCATTTGCCT	399	DebRoy *et al.* (2010)
	wzy-R	GATGACATT ACCCAAACCAGAACC		

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Table 2 (Continued)

O group	Gene	Primer sequence (5′–3′)	Amplicon (bp)	Reference
	wzx-F	TGGGCTTGATTGGCGGTAGTGTAT	701	DebRoy *et al.* (2010)
	wzx-R	AGGTCCCATCCAAGCGGACATTAT		
O149	*wzy*-F	TTTGGTGCAGATACTCAGA	709	Han *et al.* (2007)
	wzy-R	GAACAATAGATGCGATACAA		
O157	*wzy*-F	TCAGCGGCTAAGTTGATT	861	Han *et al.* (2007)
	wzy-R	ATTTGCTCCCATGTCTCC		
O158	*wzx*-F	CGTCCGAAGCAAAGATGA	624	Wang Q *et al.* (2010a)
	wzx-R	ATACTGAATATGCCCGTGA		
	wzy-F	CCCAGAACAATAACACCG	700	Wang Q *et al.* (2010a)
	wzy-R	TATTTCGTTCTGAAGCTGAC		
O165	*wzx*-F	TCATTTAGAGCCCTCACTTT	1043	Wang Q *et al.* (2010a)
	wzx-R	GAATCACGCTTTAACGCATA		
	wzy-F	TTTATGGTTATTCAGGGCAGAT	896	Wang Q *et al.* (2010a)
	wzy-R	AATAATACCATAGGGTCCAAA		
O172	*wzx*-F	TGTTGATGGTTAAAATA	704	Guo *et al.* (2004)
	wzx-R	CATAATAAGGACATGAC		
	wzy-F	ACTAACAACATCAGTAAA	860	Guo *et al.* (2004)
	wzy-R	CCAGCAACCCCCAAAACCC		
O173	*wzx*-F	ACTTTGCCATATCTTGTTCG	345	Wang Q *et al.* (2010a)
	wzx-R	TTCCTTTCCCTGAAACAACC		
	wzy-F	TTCAAAGTGCTCTGGAGGGA	606	Wang Q *et al.* (2010a)
	wzy-R	TGGCTGAGACTTGACTATTTTC		
O174	*wzx*-F	TCTAGGACCTGTAGAACA	656	Beutin *et al.* (2005a)
	wzx-R	GTAGTTGATCTGAGCGAT		
	wzy-F	TATGGGTCCTATTACTTTC	759	Beutin *et al.* (2005a)
	wzy-R	GTATCGGAGATCATTATTA		
O177	*wzx*-F	GTTGCGTTGCCTGCTGTA	680	Beutin *et al.* (2005a)
	wzx-R	GGTAAAGCCCTATCATCC		
	wzy-F	TTTTATTAGGGTCAGGAG	491	Beutin *et al.* (2005a)
	wzy-R	CACAACGACGGATTATCA		

Detection of O groups by microarrays

Microarrays have been developed for identification of *E. coli* O groups (Liu and Fratamico, 2006) and for serotyping of enterotoxigenic *E. coli* (Wang *et al.*, 2010b). Model DNA microarrays developed by Liu and Fratamico (2006) employed either oligonucleotides or PCR products spotted onto arrays followed by hybridization with labeled long PCR products representing the entire O-antigen gene cluster of specific *E. coli* serogroups. This microarray format can be expanded to target all of the *E. coli* O serogroups, and potentially also identify the H type by targeting genes encoding for the *E. coli* H flagellar antigens (e.g., *fliC*), as well (Wang *et al.*, 2003). DNA microarrays have been developed for detecting strains involved in bovine diarrhea and septicemia (Liu *et al.*, 2010) and for the detection of *E. coli* causing post-weaning diarrhea and edema disease in pigs (Han *et al.*, 2007). Li *et al.* (2006) developed a serotype-specific DNA microarray for identification of serogroups *E. coli* O55, O111, O114, O128 and O157.

Future directions

Although the DNA sequences of 96 O antigen gene clusters have already been reported, the nucleotide sequences of another 78 O groups are still being pursued. Comparative genomics of the O-antigen gene clusters and significance of genetic aberrations in the clusters will be apparent once all the clusters are sequenced. A better understanding of the genetic loci encoding all O-antigens that confer pathogenicity would lead to a more comprehensive view of the structural patterns and biosynthetic pathways used to build such antigens and of their role in pathogenesis. The genomic sequences will enable researchers to: (a) determine the role and relevance of genes encoding the O antigen that may be implicated

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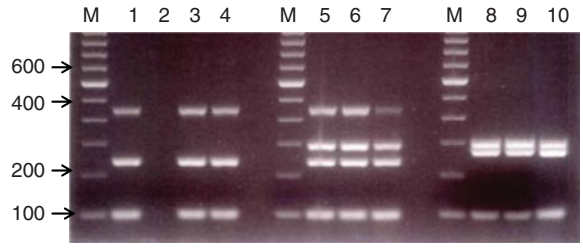

Fig. 3. Multiplex PCR detection of *E. coli* O22:H1 E14a targeting the *wzx* and *wzy* genes in spiked dog feces and ground beef. Lane M, molecular weight markers. Lane 1, PCR products for O22 *wzx* (458 bp) and O22 *wzy* (246 bp) from standard reference strain E14a (positive control) and 16S rRNA internal 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 105 CFU/g of dog feces. Lane 4, dog feces spiked with *E. coli* O22 at 106 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 2 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). Reproduced with permission from Fratamico *et al.* (2009a).

as determinants of pathogenicity and disease specificity in humans and animals; (b) evaluate the molecular mechanisms for host specificity associated with different O groups in human and animal infections; (c) elucidate the molecular mechanisms of host adaptation and immune system evasion; (d) identify specific genes and proteins suitable for use in the development of the next generation of diagnostic, therapeutic and immunoprophylactic agents.

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