Molecular Serotyping of Escherichia coli O26:H11

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Serotyping is the foundation of pathogenic Escherichia coli diagnostics; however, few laboratories have this capacity. We developed a molecular serotyping protocol that targets, genetically, the same somatic and flagellar antigens of E. coli O26:H11 used in traditional serotyping. It correctly serotypes strains untypeable by traditional methods, affording primary laboratories serotyping capabilities.

Human infections with Escherichia coli O26:H11 and O26: H-untypeable (H-) or H-nonmotile (NM) strains are associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (2, 33). Depending on virulence factors, E. coli O26 is classified as enterohemorrhagic (EHEC) or enteropathogenic (EPEC). The E. coli O26 serotype, first reported to cause pediatric EPEC diarrhea (18), has been isolated from EHEC outbreaks in Europe (1, 13, 15, 31), South America (22, 28), and Japan (10, 11); from sporadic cases in Canada (12), Australia (7), and the United States (4); and from sick (14, 21) and healthy (20, 25, 32) livestock. E. coli O26 is the most frequently isolated non-O157 Shiga-toxigenic E. coli (STEC) associated with human clinical illness (3), and E. coli O26:H11 is the clinically most important and epidemiologically most predominant EPEC and EHEC O26 serotype (2, 33).

Conventional E. coli O:H serotyping by agglutination of somatic and flagellar antigens by the use of anti-E. coli polyclonal antisera is time consuming, expensive, and available only in a small number of reference laboratories (3, 33). E. coli O:H serotyping of pathogens may be required, however, for proper diagnosis and treatment, to maximize an isolate’s usefulness for surveillance and to determine overall disease trends (33). PCR-based methodologies to detect or identify pathogenic E. coli O26 strains have targeted virulence genes such as Shiga toxin (stx) (9) and intimin (eae) (12, 27), flagellar H-antigen genes fliC-fliA (17), and the O-antigen O26 wzx genes (5, 16, 23). PCR methods targeting O26 O-antigen or H11 fliC genes could substitute for serotyping (5, 24). We therefore developed a molecular serotyping method to target the somatic and flagellar antigens and to allow clinical laboratories to accurately serotype E. coli as O26 and/or H11 by multiplex PCR (mPCR).

A 12-kb region of the E. coli O26:NM O-antigen operon from bovine fecal isolate SB6629 was sequenced (accession number AY763106); this region is identical to the corresponding region of the O26 O-antigen operon sequence (AF529080) reported by D’Souza et al. (6). Twenty sets of E. coli O26 O-antigen specific primers were synthesized and evaluated against a small panel of E. coli O26 and non-O26 E. coli isolates. One primer set, w2x-w2yO26F and w2x-w2yO26R, targeting the O26 w2x-w2y genes (O-antigen flippase and polymerase, respectively), had 100% sensitivity and specificity and a suitable amplicon size.

The E. coli H7 flagellum gene sequence is heterogenous, and fliC111 sequence of the O157 serogroup is distinct from fliC111 sequence of other O serogroups (29). To determine H11 flagellum sequence heterogeneity across different O serogroups (35), the fliC111 genes of 20 H11 E. coli isolates comprising five O serogroups were sequenced (accession numbers AY906918 to AY906937). Eighteen of these sequences were identical to those previously reported (34, 35). Two E. coli O26:H11 strains had one and two nonsynonymous substitutions, respectively, compared to the consensus fliC111 sequence. Thus, we chose to use fliC111 primers designed by Wang et al. (30) for our assay.

The primers for E. coli O26:H11 molecular serotyping were w2x-w2yO26F (5′-AAATTAGAAGCGCGTTTCATC), w2x-w2yO26R (5′-CCCAGCAAGGCCTATTGACT), fliCRH11-1 (5′-ACTGTTAACGTAGATAGC) (30), and fliCRH11-2 (5′-TCAATTTCGAGAAATAC) (30). Cells were prepared by diluting overnight Trypticase soy broth cultures 1:10 with reagent grade water. The 20 μl PCR used 1 μl of template and 0.5 U HotStarTaq (QIAGEN, Valencia, CA). The PCR mix contained concentrations of 500 μM per nucleotide, 600 mM for each primer, and 4.5 mM MgCl2. Thermocycler conditions were 95°C for 15 min, 35 cycles of 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s, and a final elongation of 72°C for 10 min. Amplicons were visualized by electrophoresis on a 2% agarose gel run at 100 V for 2 h, stained with ethidium bromide, and digitally photographed. Isolates positive for the O26 and H11 alleles displayed bands of 596 bp and 224 bp, respectively (Fig. 1). We applied the E. coli O26:H11 mPCR to three diverse bacterial isolate panels to evaluate assay performance. Panel I was composed of 344 isolates: 322 diverse E. coli of known O and/or H serotype (including 31 E. coli O26:H11 strains) of human, animal, insect, and environmental origins, and 22 non-E. coli. This panel was used to generate mPCR diagnostic sensitivity and specificity estimates. (A detailed list of the strains used is available at http://www.ars.usda.gov/sp2UserFiles/Place/54380570/ARHU/Ecoli/AEM_Durso_isolate_list.pdf). The O26 and H11 serotype status of 42 E. coli strains comprising panel II was tested blindly (without knowledge of their conventional O:H serotype). Panel III, a subset of panel...
I composed of 35 E. coli O26:NM (34 eae positive, 1 eae negative) and 11 O26:H- (10 eae positive, 1 eae negative) strains, was used to estimate the proportion of E. coli O26:NM/H- strains that possess the fliC_H11 gene without expressing the H11 antigen. Although all reactions were run in the multiplex format, assay performance was measured separately for the O26 and H11 primer sets. Additionally, E. coli O26 strains were serologically confirmed as O26 by enzyme immunoassay using an anti-E. coli O26 murine monoclonal antibody (26), and E. coli O26 and H11 strains were assayed for the presence of stx1, stx2, and eae genes as previously described (19) (Table 1).

The E. coli O26/H11 mPCR assay was 100% sensitive and specific based on reactions with the 344 panel I bacteria (Table 1). The assay correctly determined the O26 and H11 serotype of the 42 blind-panel E. coli strains (Table 2). All 35 E. coli O26:NM and 10 of 11 E. coli O26:H- strains in panel III were fliC_H11 positive. These results are consistent with the findings of Zhang et al. (34), who noted that eae-positive E. coli O26:H- strains belong to an H11 clonal complex. The fliC_H11-negative E. coli O26:H- strain was eae negative and therefore not part of the H11 clonal complex. The mPCR assay also identified seven strains that had apparently been misserotyped or mislabeled with respect to O26 and/or H11 serotype. The mPCR and serotyping results for these seven strains initially appeared discordant. However, when these isolates were rerotyped by the E. coli Reference Center (University Park, PA), the revised results agreed with those of the mPCR O26/H11 assay. Importantly, molecular serotyping methods generate genotypic information, in contrast to traditional serotyping, which generates phenotypic (surface antigen expression) information. Thus, our E. coli O26/H11 mPCR permits serotyping of “antigenically silent” strains which are O or H untypeable by conventional serotyping techniques. For example, our PCR assay serotyped rough lipopolysaccharide (O-antigen-free) colonies of blind-panel isolate TW07187 as O26, and identified the fliC_H11 gene in many NM and H- E. coli strains, in both O26 and non-O26 STEC.

In conclusion, the E. coli O26/H11 mPCR assay accurately determined O26 and H11 serotypes of 364 conventionally serotyped E. coli strains by targeting the genes that encode the same somatic and flagellar antigens used in traditional serotyping. Furthermore, the mPCR assay serotyped E. coli isolates (such as rough O26 and nonmotile H11 strains) which were untypeable by conventional methods. Third, we found that 45 out of 46 STEC and non-STECE. coli strains were fliC_H11 positive, analogous to the case of STEC O157:NM strains, which almost always possess the fliC_H11 gene (8). Finally, the assay allowed for confirmation of E. coli O26 and H11 serotype.

Traditional E. coli O:H serotyping, which detects surface antigen expression, will remain important, but application of molecular serotyping techniques that detect their genetic analogues (counterparts) have some relative advantages. While molecular E. coli O:H serotyping may not be appropriate for all bacteriologic investigations, it is well suited for clinical diagnostic, research laboratory, or epidemiologic investigations.

### TABLE 1. Sensitivity and specificity estimates of E. coli O26 and H11 mPCR compared to traditional serotyping for 344 panel I bacteria (222 E. coli and 22 non-E. coli)*

<table>
<thead>
<tr>
<th>Measure</th>
<th>No. of strains used</th>
<th>Estimate</th>
<th>95% Confidence interval</th>
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<tr>
<td>O26 sensitivity</td>
<td>48 E. coli O26 eae+, stx+</td>
<td>1.00</td>
<td>0.96–1.00</td>
</tr>
<tr>
<td></td>
<td>29 E. coli O26 eae+, stx-</td>
<td></td>
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<tr>
<td></td>
<td>2 E. coli O26 eae-, stx+</td>
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</tr>
<tr>
<td></td>
<td>2 E. coli O26 eae-, stx-</td>
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</tr>
<tr>
<td></td>
<td>238 non-O26 E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 non-E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O26 specificity</td>
<td>26 E. coli H11 eae+, stx+</td>
<td>1.00</td>
<td>0.99–1.00</td>
</tr>
<tr>
<td></td>
<td>10 E. coli H11 eae+, stx-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>142 non-H11 E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 non-E. coli</td>
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* Data compiled from multiplex PCR reactions. See isolate list for data on which strains were used in each analysis (http://www.ars.usda.gov/sp2UserFiles/Place/54380570/AHRU/E.coli/AEM_Durso_isolate_list.pdf).

Non-O26 E. coli includes 238 isolates of 111 different O serotypes and excludes three isolates for which O serotype data were unavailable.

Non-E. coli includes 22 isolates of 17 closely related genera.

Non-H11 E. coli includes 142 isolates of 51 different H serotypes and excludes all strains for which H serotype data were missing, all strains that did not react with any of the standard H antisera, and all nonmotile strains.
The E. coli O26:H11 mPCR assay described here should allow laboratories to generate E. coli O26 and H11 serotype data using routine PCR techniques and readily available equipment, since fewer laboratories have the capacity for serotyping non-O157 STEC (33). The assay was designed to allow for the addition of other PCR targets, such as stx or eae. We optimized this assay for use with a pure bacterial culture; as with traditional serotyping, it is not appropriate for application to complex sample matrices with mixed microbial flora.

**Nucleotide sequence accession numbers.** The nucleotide sequence for E. coli O26 for the NM O-antigen operon from bovine fecal isolate SB629 is deposited in GenBank as AY763106. Nucleotide sequences for 20 E. coli BIbCH1 strains are as follows: strain ATCC 35401, O serotype 78, AY906918; strain DEC 10J, O serotype 70, AY906919; strain P1331, O serotype 26, AY906920; strain H311B, O serotype 26, AY906921; strain 89-491, O serotype 26, AY906922; strain 88-353, O serotype 26, AY906923; strain 88-157, O serotype 26, AY906924; strain DEC 9E, O serotype 26, AY906926; strain DEC 9D, O serotype 26, AY906927; strain DEC 9A, O serotype 26, AY906928; strain DEC 10E, O serotype 26, AY906929; strain DEC 10D, O serotype 26, AY906930; strain DEC 10C, O serotype 26, AY906931; strain DEC 10B, O serotype 26, AY906932; strain Su 4321, O serotype 13, AY906933; strain 88-41, O serotype 111, AY906934; strain DEC 10A, O serotype 26, AY906935; strain DEC 8D, O serotype 26, AY906936; strain CL5, O serotype 26, AY906937.

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Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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