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Note

## Molecular serotyping of *Escherichia coli* O111:H8

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### Abstract

Accurate *Escherichia coli* serotyping is critical for pathogen diagnosis and surveillance of non-O157 Shiga-toxigenic strains, however few laboratories have this capacity. The molecular serotyping protocol described in this paper targets the somatic and flagellar antigens of *E. coli* O111:H8 used in traditional serotyping, and can be performed routinely in the laboratory.

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**Keywords:** *E. coli*; *E. coli* O111:H8; O111:H8; Serotyping; Molecular serotyping

Serotyping has long served to help distinguish between different *Escherichia coli*. Of the 181 numbered *E. coli* O antigens, only a small subset is associated with disease in humans. *E. coli* with the O111 serotype causes both enteropathogenic and enterohemorrhagic diseases in humans (Nataro and Kaper, 1998). Enteropathogenic *E. coli* (EPEC) O111 is a classic cause of diarrhea in children, especially in the developing world. Enterohemorrhagic *E. coli* (EHEC) O111, which typically carries at least one Shiga-toxin gene, is one of the most common non-O157 causes of bloody diarrhea and hemolytic–uremic syndrome (HUS) in the United States (Brooks et al., 2005), and also causes disease in Europe (Karch et al., 1999), Asia (Jeon et al., 2006; Kato et al., 2005), and Australia (Paton et al., 1996). Numerous outbreaks have been attributed to this pathogen (Bettelheim and Goldwater, 2004; Brooks et al., 2004; Jeon et al., 2006; Kato et al., 2005). There are a limited number of H antigens associated with both EPEC and EHEC O111, including H8 and H– (Bettelheim, 2003). The O111:H8 serotype has been implicated as a cause of enterohemorrhagic diarrhea and was shown to be the source of the only reported EHEC O111 outbreak in the United States which occurred at a Texas cheerleading camp (Benenson, 1995; Brooks et al., 2005). *E. coli* O111:H8 can also be isolated from livestock, including cattle (Jeon et al., 2006), and the

epidemiology of this serotype in cattle and the cattle production environment remains unclear.

In conventional serotyping, an anti-*E. coli* polyclonal antiserum is used to agglutinate somatic and flagellar antigens. However, the resources needed to reliably perform *E. coli* O– and H– antigen serotyping are only available in a limited number of reference laboratories (Bettelheim, 2003). Some PCR-based methods indirectly identify *E. coli* O111 via virulence genes or intimin subtypes (Blanco et al., 2004; Jenkins et al., 2003; Sharma, 2002). While these methods appear to work well, the PCR targets can only serve as markers for serotype, since the actual genes involved in antigen expression are not assayed. However, PCR methods targeting *E. coli* O– or H– antigen genes directly could substitute for serotyping (DeRoy et al., 2004; Durso et al., 2005; Prager et al., 2003), and PCR primer sets targeting the *E. coli* O111 and the H8 antigens have been developed (Paton and Paton, 1998; Wang et al., 2003). We have combined these primers in a single molecular serotyping assay, and validated the multiplexed assay with both EPEC and EHEC strains.

Primers for *E. coli* O111:H8 molecular serotyping were: O111 F (AGA GAA ATT ATC AAG TTA GTT CC) and O111 R (ATA GTT ATG AAC ATC TTG TTT AGC), which are specific for the *wbdl* portions of the *rfb*<sub>O111</sub> (O-antigen-encoding) gene cluster (Paton and Paton, 1998) and H8F (AGT GAC CTG ATT TCT AAA) and H8R (TTC AAT GGA GCC TTT GTC), which code for the *fliC*<sub>H8</sub> flagellar protein (Wang et al., 2003). Cells were prepared by diluting overnight Trypticase Soy Broth

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cultures 1:10 with reagent grade water. The 30  $\mu$ l PCR reaction recipe was determined empirically, and the optimized reaction conditions used 120  $\mu$ M of each nucleotide, 500 nM of each primer, 4.5 mM MgCl<sub>2</sub>, 1  $\mu$ l of template (diluted overnight culture) and 0.5 unit HotStarTaq (Qiagen, Valencia, CA). Thermocycler conditions were determined through a series of temperature gradient experiments. Final thermocycler conditions were: 95 °C for 15 min, 35 cycles of 94 °C for 60 s, 53 °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, stained with ethidium bromide, and digitally photographed. The O111 amplicon was 406 bp and the H8 amplicon was 238 bp (Fig. 1). In order to validate that the PCR amplicons were *rfb*<sub>O111</sub> and *fliC*<sub>H8</sub>, all positive PCR bands were sequenced. Amplicons were treated with exonuclease (USB) and precipitated using ethanol (Smith et al. 2000). The pellet was suspended in 1 $\times$  DYEnamic ET termination cycle sequencing reagent (Amersham Bioscience, Pittsburgh, PA) and subjected to 25 cycles of 96 °C for 30 seconds, 50 °C for 1 minute, and 60 °C for 4 minutes, using the amplification primers to sequence. Following a second ethanol precipitation, the pellet was suspended in formamide (PE Biosystems, Foster City, CA) and sequenced using an ABI 3730xl DNA analyzer (PE Biosystems). The sequencing reads were queried against the BLAST nr database (NCBI), which confirmed the amplicon's sequence as being either *E. coli wbdI*<sub>O111</sub> or *fliC*<sub>H8</sub>.

Molecular serotyping assay sensitivity and specificity estimates were determined using a panel of 259 diverse *E. coli* of known O and/or H serotype (including 54 *E. coli* serotyped as O111 and 31 serotyped as H8) of human, animal, and insect origins, and 23 non-*E. coli*. Sensitivity is the proportion (range 0.0–1.0) of true positives (as measured by the gold standard, in this case traditional serotyping) classified as positive by the test (molecular serotyping assay). High sensitivity estimates indicate a low number of false negative results. Diagnostic specificity is the proportion of true negatives

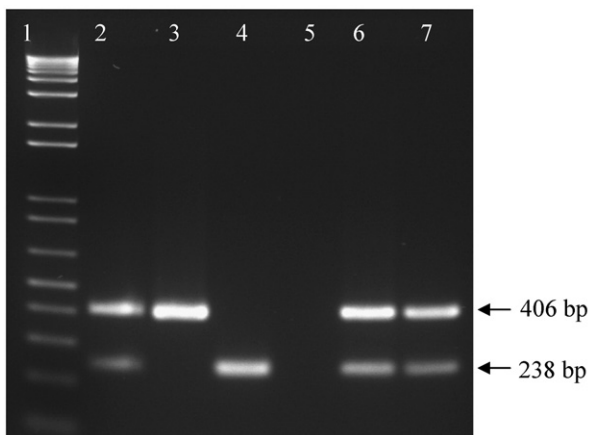


Fig. 1. Molecular serotyping of *E. coli* O111:H8 by multiplex PCR. Lane 1, molecular marker (100 bp); lane 2, Dec 8B (O111:H8 by conventional serotyping); lane 3, Dec 12B (O111:H2 by conventional serotyping); lane 4, Dec 10G (O128:H8 by conventional serotyping); lane 5, Bi 7458-41 (O6:H1 by conventional serotyping); lane 6, BE 00749 (O111:HNM by conventional serotyping); and lane 7, TWO4579 (O111:H- by conventional serotyping).

Table 1

Sensitivity and specificity estimates of *E. coli* O111 and H8 molecular serotyping<sup>a</sup> compared to conventional serotyping for 253 bacteria (231 *E. coli* and 22 non-*E. coli*)

Measure	Strains used	Estimate	95% CI
O111 specificity (n=228)	205 non-O111 <i>E. coli</i> <sup>b</sup> 23 non- <i>E. coli</i> <sup>c</sup>	1.00	0.99–1.00
O111 sensitivity (n=54)	54 <i>E. coli</i> O111 <sup>d</sup>	0.98	0.90–0.99
H8 specificity (n=147)	124 non-H8 <i>E. coli</i> <sup>e</sup> 23 non- <i>E. coli</i> <sup>c</sup>	1.00	0.97–1.00
H8 sensitivity (n=32)	32 <i>E. coli</i> H8 <sup>f</sup>	0.96	0.84–0.99

<sup>a</sup> Data compiled from multiplex PCR reactions.

<sup>b</sup> Non-O111 *E. coli* includes 205 isolates of 173 different O serotypes.

<sup>c</sup> Non-*E. coli* includes 23 isolates of 17 closely related genera.

<sup>d</sup> Includes 54 *E. coli* O111 isolates of 9 different H serotypes, and excludes all strains for which H serotype data was missing, all strains that did not react with any of the standard H antisera, and all non-motile strains.

<sup>e</sup> Non-H8 *E. coli* includes 124 isolates of 39 different H serotypes, and excludes all strains for which H serotype data was missing, all strains that did not react with any of the standard H antisera, and all non-motile strains.

<sup>f</sup> Includes 32 isolates of 31 different O serotypes.

(as measured by traditional serotyping) classified as negative by the test (molecular serotyping assay). High specificity estimates indicate a low number of false positive results. The O111 and H8 components of assay performance were measured separately. The proportion of *E. coli* O111:NM/H- strains that possess the *fliC*<sub>H8</sub> gene without expressing the H8 antigen was estimated based on data collected from seven *E. coli* O111:H- (H antigen does not match any of the standard sera), and 17 O111:HNM (NM = non-motile, H antigen not expressed) strains.

Compared to conventional serotyping, results (Table 1 and supplementary data at <http://www.ars.usda.gov/SP2UserFiles/person/35149/Supplements/DursoJMMSupDataJan222007.pdf>) indicate that the *E. coli* O111:H8 molecular serotyping assay was 100% specific for both the O111 and H8 antigens (no false positives) and had a sensitivity of 0.98 for the O111 antigen (95% CI 0.90–0.99) and 0.96 for the H8 antigen (95% CI 0.83–0.99). Sequencing of the O111 and H8 positive PCR bands confirmed them to be *rfb*<sub>O111</sub> and *fliC*<sub>H8</sub>, respectively, in all cases. There was a single EPEC O111:HNM strain (strain 5720 B170 received in 1996 from the Centers for Disease Control and Prevention) that was positive by conventional serotyping, but negative in the molecular serotyping (false negative). The only false negative H8 isolate was an O102:H8 strain that is used by the CDC as an O102 reference strain. The H8 conventional serotype was confirmed in our laboratory using reference sera obtained from the Statens Serum Institut (Copenhagen, Denmark). It is possible that these false negatives result from sequence diversity under the primer sites. There were 11 of 17 (65%) *E. coli* O111:NM and four of eight (50%) *E. coli* O111:H- that gave a positive band for the H8 gene *fliC*<sub>H8</sub> with molecular serotyping.

Like conventional serotyping, this assay is designed and optimized for use with pure bacterial cultures, and it is not appropriate for primary isolation or detection of *E. coli* O111 or *E. coli* H8 from complex sample matrices. Unlike conventional

serotyping, which is based on phenotypic information about antigen expression, molecular serotyping generates information on the presence or absence of the gene that codes for the antigen. For this reason, molecular serotyping may not be appropriate for all bacteriologic investigations, especially when phenotypic expression data is needed. However, molecular serotyping does permit serotyping of “antigenically silent” strains that cannot be typed using conventional methods, such as the H- and non-motile H8 strains examined in these experiments.

In conclusion, the *E. coli* O111:H8 molecular serotyping assay targets the same *E. coli* O111 and H8 somatic and flagellar antigens used in conventional serotyping and is both sensitive and specific compared to conventional serotyping methods. Although traditional *E. coli* O:H serotyping will remain the gold standard for *E. coli* O111:H8 diagnosis, the molecular serotyping assay described here has some relative advantages including the ability to serotype isolates that are untypable by conventional methods. In addition, the *E. coli* O111:H8 molecular serotyping assay described here should allow laboratories to reliably and cost-effectively generate *E. coli* O111 and H8 serotype data using routine PCR techniques and readily available laboratory equipment.

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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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