Sensitivity of an Immunomagnetic-Separation-Based Test for Detecting Escherichia coli O26 in Bovine Feces

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The recovery of Escherichia coli serogroup O26 from cattle has been reported for more than 50 years (2, 17) from a wide range of countries (5, 15, 18, 22, 23). In a human context, E. coli O26 has been associated with infantile diarrhea (21), and verocytotoxin-producing strains have been identified as a cause of hemolytic-uremic syndrome (13). E. coli O26 was the most common serogroup recovered from human verocytotoxin-producing E. coli (VTEC) infections in Italy between 1995 and 2001 (20), was the second most frequent serogroup among human VTEC isolates in Scotland during 2003 (14), and is responsible for 20% of human clinical enterohemorrhagic E. coli infections in Japan (11). Domestic ruminants, including cattle and sheep, are significant reservoirs of VTEC and are thought to be a major source of human non-O157 infection (24).

A reliable test for E. coli O26 has to detect an appropriate concentration of its target with high sensitivity and specificity in the presence of associated microflora. Several methods to achieve this end have been described (7, 10, 19), including the use of antibody-coated immunomagnetic-separation (IMS) beads (18, 23), though there has only been a limited evaluation of IMS-based tests for E. coli O26 (12, 18). We felt that it was desirable to evaluate a previously used IMS-based test for E. coli O26 (17, 18) more thoroughly to help validate cattle prevalence estimates. This report describes our approach to estimating the sensitivity of this test.

MATERIALS AND METHODS

Outline. The recovery of E. coli O26 from six fecal pats artificially inoculated with six different E. coli O26 strains at concentrations ranging from 10^1 to 10^6 CFU g^-1 feces was measured. E. coli was recovered using a test that involves the enrichment of fecal microflora in buffered peptone water, the selective concentration of E. coli O26 using antibody-coated immunomagnetic-separation beads, the identification of E. coli colonies on Chromocult tryptone bile X-glucuronide agar, and confirmation of the serogroup with E. coli O26-specific antisera using slide agglutination. The effective dose of E. coli O26 for an 80% test sensitivity (ED80) was 1.0 x 10^4 CFU g^-1 feces (95% confidence interval, 4.7 x 10^3 to 2.4 x 10^5). Differences in test sensitivity between different E. coli O26 strains and fecal pats were also observed. Individual estimates of ED80 for each strain and fecal pat combination ranged from 4.2 x 10^2 to 4.8 x 10^6 CFU g^-1. These results suggest that the test is useful for identifying individuals shedding a large number of E. coli O26 organisms or, if an appropriate number of individuals in a herd are sampled, for identifying affected herds. The study also provides a benchmark estimate of sensitivity that can be used to compare alternative tests for E. coli O26 and a methodological approach that can be applied to tests for other pathogenic members of the Enterobacteriaceae and other sample types.

Origin and preparation of strains. Five of the six E. coli O26 strains used in this experiment had been recovered previously without using IMS from bovine feces sampled from around Scotland and had been stored at –80°C (laboratory strain codes C1414.1, C1991, C026.4, C683.1, and 56C280/2). They are characterized by different pulse-field gel electrophoresis profiles, and while some change cannot be discounted, they are essentially unmodified field strains. The sixth strain (laboratory strain code UA3552NAR) is a nalidixic acid-resistant laboratory-adapted strain of unknown origin obtained from Iain Ogden, University of Aberdeen. Five of the strains possess the vtx gene, one strain has an additional vtx2 gene, three strains have elbV4, and all six strains have eae. Each strain was individually cultured overnight in 20 ml BPW (Oxoid, Basingstoke, United Kingdom) at 37°C. An approximate concentration for each culture was estimated from its optical density at 570 nm, and a series of 10-fold dilutions ranging from approximately 10^6 to 10^10 CFU ml^-1 was prepared for each strain in phosphate buffered saline, pH 7.4 (PBS; Sigma-Aldrich, Poole, United Kingdom), for inoculation.

Origin and preparation of fecal pats. A sample was taken from each of 11 fresh bovine fecal pats from separate farms near Inverness during November 2003. These samples were stored at 4°C. Six of the fecal pats were from enclosures of housed cattle and the remaining five from grazing cattle. The ages of the animals from which pats were collected are unknown. Ten 1-g subsamples were taken from each stirred fecal pat sample and were screened for endogenous E. coli O26 within 48 h of collection by using the procedure for recovery of E. coli O26 described below.

Interference with E. coli O26 growth by feces. Growth interference for each of the six E. coli O26 strains by each of the 11 fecal pats was evaluated. For each E. coli O26 strain, two 10^- by 10^- cm plates containing TBX agar (Merck, Darmstadt, Germany) were flooded with a 0.5 McFarland standard solution of the respective strain prepared in sterile saline. Excess solution was poured off and the plate dried. Six 8.5-mm-diameter wells were bored into each plate, and a subsample of...
TABLE 1. Observed recovery of E. coli O26 by culture on TBX after IMS from inoculated bovine feces

<table>
<thead>
<tr>
<th>Concentration of inoculated E. coli O26 in feces (CFU g⁻¹)</th>
<th>No. of positive subsamples tested</th>
<th>% Positive subsamples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>5/36</td>
<td>14%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>3/34</td>
<td>9%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁺</td>
<td>7/36</td>
<td>19%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>18/35</td>
<td>51%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>21/35</td>
<td>60%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>29/34</td>
<td>85%</td>
</tr>
<tr>
<td>1.6 × 10⁻¹ to 8.4 × 10⁻¹</td>
<td>33/36</td>
<td>92%</td>
</tr>
</tbody>
</table>

* The highest number of E. coli O26 inoculated into a subsample was 8.4 × 10⁴ CFU.

TABLE 2. Predicted percent probability of recovering E. coli O26 from inoculated bovine feces

<table>
<thead>
<tr>
<th>Concentration of inoculated E. coli O26 in feces (CFU g⁻¹)</th>
<th>% Probability of recovery</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁴</td>
<td>28</td>
<td>21–37</td>
</tr>
<tr>
<td>1 × 10⁵</td>
<td>56</td>
<td>46–65</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>80</td>
<td>73–86</td>
</tr>
<tr>
<td>1 × 10⁷</td>
<td>93</td>
<td>90–95</td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>98*</td>
<td>96–98</td>
</tr>
</tbody>
</table>

* Indicates the highest number of E. coli O26 inoculated into a subsample was 8.4 × 10⁴ CFU.

RESULTS

Observed percentages of inoculated fecal subsamples from which E. coli O26 was recovered are presented in Table 1. These range from 9% of subsamples containing between 1.6 and 8.4 CFU of E. coli O26 g⁻¹ of feces to 92% of those containing between 1.6 × 10⁴ and 8.4 × 10⁴ CFU g⁻¹.

Predicted percent probabilities of recovering E. coli O26 from inoculated fecal subsamples are presented in Table 2. These range from 28% (CI, 21% to 37%) to 93% (CI, 90% to 95%) for subsamples containing 1 × 10⁴ and 1 × 10⁵ CFU of inoculated E. coli O26 g⁻¹ of feces, respectively. The mean ED₈₀, averaged across strains and fecal pats, was 1.0 × 10⁶ CFU of inoculated E. coli O26 g⁻¹ feces (CI, 4.7 × 10⁵ to 2.4 × 10⁶). Estimates of ED₈₀ for each strain and fecal pat combination range from 4.2 × 10⁵ to 4.8 × 10⁵ CFU g⁻¹, with first and third quartiles of 1.1 × 10⁵ and 1.0 × 10⁵ CFU g⁻¹.

E. coli O26 was recovered from a minimum of 12 and a maximum of 21 of the 29 fecal pat-concentration combinations tested for each strain. Differences in recovery between strains are statistically very highly significant (χ² = 122.45; P < 0.001). Estimates of ED₈₀ for individual strains range from 4.7 × 10⁵ to 2.8 × 10⁶ CFU g⁻¹.

E. coli O26 was recovered from a minimum of 8 out of 31 and a maximum of 24 of the 30 strain-concentration combinations tested for each fecal pat. A preliminary statistical analysis, omitting an animal housing effect, suggested that differences in recovery from fecal pats from housed and grazing animals may partly explain this. E. coli O26 was recovered from 68 of 92 (74%) and 41 of 90 (46%) inoculated subsamples from grazing and housed animals, respectively, a difference that approaches statistical significance (F₁,5 = 6.21; P = 0.067). This possible systematic effect does...
not account for all the variation, however, and differences between fecal pats from the same management system remain statistically very highly significant (χ² = 30.75; P < 0.001). Estimates of ED₉₀ for individual fecal pats range from 9.1 × 10⁵ to 1.7 × 10⁶ CFU g⁻¹.

Control results for the experiment are as follows. First, *E. coli* O26 was not recovered from any of the negative-control subsamples. Second, although interference with the growth of *E. coli* O26 by feces was observed for 7 of the 36 strain–fecal pat combinations, involving 4 strains and 3 fecal pats (data not shown), there was no evidence of an association between the recovery of *E. coli* O26 from inoculated fecal subsamples and the pattern of growth-interference (F₁,₁₇₈ = 1.24; P > 0.10).

Third, *E. coli* O26 strains with PCR banding patterns different from those inoculated were recovered from six subsamples. These involved five fecal pats, three strains, and inoculum concentrations ranging from 1.6 × 10⁶ to 3.4 × 10⁴ CFU g⁻¹. The PCR banding patterns of isolates from four of the subsamples were consistent with other inoculated strains, while the other two isolates were different. As mentioned above, these were excluded from the statistical analysis. Finally, there was no evidence of a difference in recovery between laboratory operators (F₁,₁₇₈ = 1.12; P > 0.10).

**DISCUSSION**

This report describes differences in the recovery of inoculated *E. coli* O26 from bovine feces for different strains and fecal pats. Differences can be explained as being due either to variation in test sensitivity or to interactions between inoculated *E. coli* O26 strains and fecal growth inhibitors. The lack of an association between the recovery of *E. coli* O26 and the pattern of growth-interference suggests that the differences are due to variation in test sensitivity. Previously published work suggesting variation between strains for an *E. coli* O157 IMS-based test (4) is consistent with these observations, although variation in recovery between fecal pats has not been previously reported. The results also raise the possibility of a difference in test sensitivity associated with fecal pats obtained from grazing and housed animals. Although the difference only approaches statistical significance, the observation is potentially important and, if confirmed, would have implications for the interpretation of statistical associations with factors such as season and diet.

The sensitivity of this test has been defined using the ED₉₀. This is the inoculated dose of *E. coli* O26 required for an 80% chance of recovering one or more *E. coli* O26 colonies from a sample using the test procedure. We use this definition because it represents a reasonable chance that a test will identify samples containing the target organism, although an ED₉₀ would be more desirable. The ED₉₀ of 1.0 × 10⁴ CFU g⁻¹ for this test is the average value for all the strains and fecal pats used in the validation. We believe that the distribution of estimates of ED₉₀ for each strain and fecal pat combination provide some guidance as to how the test performs on naturally infected samples. The range of ED₉₀ estimates for fecal pat–strain combinations from 4.2 × 10⁷ to 4.8 × 10⁴ CFU g⁻¹ compares to published estimates of the concentration of VTEC O26 in the feces of naturally infected cattle, ranging from less than 1 × 10⁸ to 1 × 10⁹ CFU g⁻¹ (7, 8, 9, 23). The utility of this test is therefore restricted to identifying individuals shedding a large number of *E. coli* O26 organisms or, if an appropriate number of individuals in a herd are sampled, to identifying affected herds.

Two observations surprised us, and it is important for future validation work to describe these. The first is the recovery from six subsamples of *E. coli* O26 strains with PCR banding patterns different from those inoculated. The second is the recovery from five subsamples of *E. coli* O26 inoculated with between 1.0 × 10⁻⁶ to 8.4 × 10⁻⁴ CFU, particularly given that we would expect the test to have a low sensitivity at these levels. Possible explanations for these observations include crossover contamination and/or the presence of undetected populations of endogenous *E. coli* O26 in the samples, although the recovery of colonies with different PCR banding patterns may be a consequence of rapid chromosomal change, perhaps similar to that observed for *E. coli* O157 (1). We are reassured by the failure to recover *E. coli* O26 from any of the 36 negative controls, which suggests that these unexpected observations do not have a major impact on the results.

An advantage of this validation procedure is that it evaluates the performance of a test from the individual performances of a range of strains in different fecal pats. However, there are also limitations with this approach. First, care has to be taken in selecting the range of inoculum concentrations. Attempts to inoculate samples at a concentration of less than 7 CFU g⁻¹ feces will result in a proportion of samples (≥0.001) receiving only carrier solution and no target organism. Care also has to be taken not to inoculate samples at too many concentrations where the test sensitivity approaches 100%, since this will result in a poor model fit and unreliable error estimates. Second, we used 6 strains and fecal pats, and while this is more than previously used on an individual rather than a pooled basis, it would be desirable to increase these to at least 10 of each for future validations. Finally, there is likely to be some concern about the use of artificially inoculated samples to estimate test sensitivity. While we cannot contest the possible validity of this, we note that a major advantage of artificial inoculation is that the number of target organisms added is known. This is not the case with alternative approaches in which fecal samples from naturally or artificially infected animals are used and where the number of target organisms has to be estimated using a less-than-perfect test procedure.

This validation experiment has not estimated the test specificity. The 36 samples inoculated with 0 CFU of *E. coli* O26 g⁻¹ could have been used for this purpose rather than as negative controls. Given our current experimental design, where the recovery of an *E. coli* O26 isolate from any one of the negative controls would have invalidated the experiment, we have not provided a quantitative estimate of specificity. However, we note that the failure to recover an isolate from any of the negative controls is an indication that the specificity of the test is high.

This study provides a benchmark estimate of sensitivity for an IMS-based test for a member of the *Enterobacteriaceae*. Although the test appears to be relatively better at detecting *E. coli* O26 than a PCR/DNA probe technique (12, 18), the results of the experiment suggest that its utility is restricted to reliably identifying individuals shedding high numbers of *E. coli* O26 organisms or, if an appropriate number of individuals
in a herd are sampled, providing estimates of herd prevalence. Both applications are useful, the first because high-shedding animals are likely to contribute disproportionately to environmental contamination and the persistence of pathogenic *E. coli* in a herd and the second because the test identifies herds where there is a current outbreak. We believe that it would be useful to apply the methodological approach described in this report to estimating the sensitivity of tests for other pathogenic members of the *Enterobacteriaceae* and other sample types.

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References


