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A monoclonal antibody (MAb) was obtained from a mouse immunized with solubilized outer membrane proteins extracted from a bovine enterohemorrhagic strain of *Escherichia coli* (EHEC), O26. The MAb produced a strong immunoblot reaction at approximately 21 kDa for an O26 strain containing the intimin gene (eae) and verocytotoxin (VT) production, but not with an O26 eae- and VT-negative strain, or O157 eae- and VT-positive strains. The MAb was used in a sandwich enzyme-linked immunosorbent assay (ELISA) format to screen strains from animal and human sources, and all reactive strains were characterized for the presence of eae and the gene encoding VT factors by PCR. The antigen was detected in a group of strains containing a high proportion of O26, the majority of which were eae positive with or without VT; these were isolated mostly from animal enteritis cases but included a small number of human enteric isolates. Nonreactors included eae-positive (with or without VT) O157 strains and one O26 strain. In a survey of mixed cultures from both animal and human enteric disease, ELISA-positive reactions were obtained from 7.1 to 11.2% of samples from bovine, porcine, ovine, and human sources. The two human O8 and ten animal O26 ELISA-reactive pure strains obtained from these samples contained six eae- and/or VT-positive strains; the other six strains lost their ELISA positivity following storage at −70°C, after which none were found to contain either eae or VT factors. The association of the antigen detected by the MAb with significant enteropathogenic *E. coli* and EHEC virulence factors in isolates from both animal and human enteric infections indicates a diagnostic potential for the assay developed.

Enterohemorrhagic *Escherichia coli* (EHEC) has been defined as a pathogenic group of strains characterized by their intimate attachment to the mammalian gut wall, leading to the production of attachment and effacement (a/e) lesions, and by verocytotoxin (VT) production (19). Another pathogenic group, the enteropathogenic *E. coli* (EPEC), is also characterized by causing a/e lesions but differs from EHEC in that it does not produce VTs. Strains from both these groups are important causes of human enteric diseases (29). EHEC strains have become prominent in recent years as causes of hemorrhagic enteritis and the hemolytic uremic syndrome. The main serogroup implicated in human disease caused by EHEC has been O157 (10), but other serogroups, in particular O26, O103, O111, and O128, have also been implicated in causing human disease (13, 22, 32).

EHEC and EPEC strains are also associated with enteric disease in cattle (5, 6, 8, 20, 21, 25, 27, 31, 33, 37). The significance of these pathogenic groups in bovine enteritis is probably underestimated, possibly because of a lack of awareness of their significance and a lack of appropriate assays for routine detection. The widespread presence of VT-producing *E. coli* strains in healthy cattle is also a complication (3, 8, 26, 35). Demonstration of VT in cultures from bovine enteritis is not sufficient to imply a causative association.

The object of the present study was to produce monoclonal antibodies (MAbs) to EHEC surface adhesion antigens, and to investigate their diagnostic application for the detection of EHEC in animal and human enteric infections. Because of an association with both human and bovine diseases, an EHEC strain of serotype O26 was selected for investigation.

**MATERIALS AND METHODS**

**Preparation of antigens.** An outer membrane (OM) preparation of *E. coli* O26 strain 4276 was prepared by the standard sarkosine extraction method (11). This strain was isolated from a calf enteritis case in Northern Ireland and was characterized as intimin (encoded by gene eae) and VT positive. Briefly, washed cells from an overnight broth culture, suspended in 0.01 M Tris HCl–0.005 M EDTA buffer, pH 7.8, were disrupted by ultrasonication. After centrifugation at 15,000 × g for 30 min to remove intact cells, the supernatant was mixed with a quarter volume of 2% (wt/vol) sodium n-laurylsarcosine (Sigma) in Tris-EDTA buffer at room temperature for 30 min and ultracentrifuged at 300,000 × g for 1 h. The resuspended pellet was reextracted with an equal volume of 2% sarcosine for 1 h at room temperature, pelleted, washed once in saline, and stored at −70°C.

Some of the washed OM was solubilized in a 6 M solution of the chaotropic agent guanidine thiocyanate (Sigma) in Tris-EDTA. Insoluble material was removed by ultracentrifugation, and the outer membrane protein (OMP) solution was dialyzed against 100 volumes of 6 M urea in Tris-EDTA buffer and stored at −70°C.

**MAbs.** A BALB/c mouse was immunized intraperitoneally with the solubilized OMP preparation of *E. coli* O26 strain 4276. Three inoculations of 100 μl, 50 μl, and 50 μl of OMP solution, each mixed with 50 μl of adjuvant (125 μg of Quil A per ml) (Superfos; DK-Vedbaek, Denmark), were given at 4-week intervals. Three days after the final inoculation, the mouse spleen cells were fused with the NSO myeloma cells at a ratio of 8:1 according to the protocol of Galfre and Milstein (12) with modifications by Teh and Wong (34). The resulting hybridomas were maintained in RPMI 1640 medium (Gibco, Paisley, United Kingdom), supplemented with 20% gamma-globulin-free horse serum (Gibco).

The cell culture fluids from actively growing hybridomas were initially screened by enzyme-linked immunosorbent assay (ELISA) in microtiter plate wells (Dynatech, McLean, Va.) coated with OM preparations of *E. coli* O26.
strains 4276 (eae and VT positive) and 1045 (eae and VT negative). The hybrid- 
omas showing specific reaction to strain 4276 antigen were cloned twice by 

limiting dilution.

Sandwich ELISA. Ascites was produced by the inapertitoneal inoculation of 

BALB/c mice with cloned hybridoma lines. The mice were primed by intraperi-
toneal inoculation of Freud’s incomplete adjuvant 2 days before cell inoculation 

(28). Ascites fluid was removed from the mice approximately 10 days later and 

stored at −20°C. Immunoglobulin was purified from the ascites fluid by caprylic 

acid precipitation (24).

The sandwich ELISA was performed on microtiter plates (Dynatech) as pre-

viously described (2–4). Briefly, 100 µl of each reagent was used per well. Optimum reagent dilutions were established by titration. The test samples 

were carried out in PTN (0.01 M phosphate-buffered saline [pH, 7.2] containing 0.04% 

Tris-HCl buffer, pH 7.2, with 0.3 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) per ml in 0.02 M Tris-HCl buffer, pH 7.2, with 0.3 µl of H2O2 (30% solution) per ml (vol/vol), was added. After incubation at room temperature for 10 min, the strips were washed with distilled water, which stopped any further reaction.

**Table 1. E. coli strains used for the preliminary testing of MAbs 2F3 and 6G5**

<table>
<thead>
<tr>
<th>Source</th>
<th>Strain</th>
<th>Pathotype*</th>
<th>Serotype</th>
<th>eae</th>
<th>VT1</th>
<th>VT2</th>
<th>2F3 ELISA</th>
<th>6G5 ELISA</th>
</tr>
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<tbody>
<tr>
<td>Bovine</td>
<td>4276</td>
<td>EHEC</td>
<td>O26</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine</td>
<td>237</td>
<td>EHEC</td>
<td>O26</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>4618</td>
<td>EPEC</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>1045</td>
<td>ETEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>E7</td>
<td>NTEC</td>
<td>O15</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>S306</td>
<td>NTEC</td>
<td>UT</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>S1378</td>
<td>NTEC</td>
<td>UT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>S784</td>
<td>EHEC</td>
<td>O157</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Bovine</td>
<td>3680</td>
<td>EPEC</td>
<td>O157</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
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<td>EHEC</td>
<td>O157</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>108</td>
<td>VTEC</td>
<td>O117</td>
<td></td>
<td></td>
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<tr>
<td>Bovine</td>
<td>286</td>
<td>ETEC</td>
<td>O141</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>335</td>
<td>ETEC</td>
<td>O139</td>
<td></td>
<td></td>
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<tr>
<td>Bovine</td>
<td>413</td>
<td>VTEC</td>
<td>O103</td>
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<tr>
<td>Porcine</td>
<td>2353</td>
<td>ETEC</td>
<td>O149</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Lapine</td>
<td>B10</td>
<td>EPEC</td>
<td>O103</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lapine</td>
<td>E22</td>
<td>EPEC</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td>Human</td>
<td>H217</td>
<td>VTEC</td>
<td>O146</td>
<td></td>
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</tr>
</tbody>
</table>

* ETEC, enterotoxigenic E. coli; NTEC, necrototoxigenic E. coli; VTEC, vero-

cytotoxic E. coli.

**RESULTS**

MAb-based sandwich ELISA. Nine of the 430 hybridomas were selected as being reactive to OM preparations of E. coli strain 4276 and nonreactive to strain 1045. Three stable clones were derived from these, one of which was no longer reactive with strain 4276. Ascites fluid was prepared with the remaining two lines, MAbs 2F3 and 6G5, and used to prepare capture and 

bionylated Mab reagents for use in sandwich ELISAs. Strain 4276 was used to optimize the assays.

Table 1 summarizes the sandwich ELISA results obtained with the collection of E. coli strains initially examined. The MAb 6G5 sandwich ELISA reacted only with the strain used to 

immunize the mouse for the hybridoma fusion. The MAb 2F3 sandwich ELISA reacted positively with only three O26 strains containing eae and/or the VT virulence factors. Neither of 

the assays reacted with Salmonella arizonae, Salmonella kentucky, 

Enterobacter spp., Klebsiella pneumoniae, Shigella flexneri, 

Pseudomonas fluorescens, Pseudomonas putida, Hafnia alvei, 

Serratia spp., Proteus vulgaris, Erwinia spp., Serratia liquefaciens, 

and Citrobacter freundii.

The sensitivity of detection for the MAb 2F3 sandwich ELISA for strain 4276 was 10^5 CFU/ml.

**Test samples.** The MAb 2F3 sandwich ELISA was used to screen various groups of E. coli strains. Table 2 summarizes the characteristics of 46 ELISA-positive and 42 ELISA-negative 

strains; these were largely from a collection of 216 strains isolated from animal enteritis cases in Northern Ireland but included two ELISA-positive bovine O111 strains from Bel-

gium, 10 ELISA-negative Northern Ireland bovine O157 

strains, and ELISA-negative O118 (n = 5), O5 (n = 2) O111 (n = 1), and O20 (n = 2) strains from Belgian cattle. The 

majority of the ELISA-positive strains were O26, although small numbers of other O-serotypes were evident. In addition, the majority of the ELISA-positive strains were either eae or 

eae and VT positive, with low numbers of eae- and VT-negative 

strains or strains only positive for VT. All of the O18 ELISA-

negative and the majority of O26 ELISA-negative strains were 

eae and VT negative, whereas both the ELISA-negative O111 

strains were eae and VT positive. Included in Table 2 are 

serotypes recognized as important causes of bovine enteritis: 

O5, O20, O111, and O118 (10), and bovine isolates of O157, all 

of which were positive for eae and/or VT.

Table 3 summarizes the ELISA results obtained with the remaining Belgian bovine strains examined, none of which was 

serotyped, but all of which were characterized for the presence
of \textit{eae}, and some for the presence of VT. Twenty-one of the 56 strains isolated from 6- to 10-week-old calves that had died with enteritis were ELISA-positive, and 35 were ELISA-negative; both groups of these strains were entirely \textit{eae} positive, with or without VT. Twenty-three out of 67 \textit{E. coli} strains isolated from two \textit{O}8 strains, from human samples, and 10 \textit{O}26 strains, one obtained from 12 of these mixed cultures; these consisted of 31 avian strains examined. China et al. (9) failed to find \textit{bfp} in animal strains that demonstrated positive reactions in the MAb 2F3 sandwich ELISA; these consisted of four \textit{O}26 strains and one \textit{O}111 strain, all of which contained the \textit{eae} and VT virulence factors; 33 of the 44 ELISA-negative strains also contained these factors. The third set of Belgian strains tested were 190 \textit{eae}-positive \textit{E. coli} strains isolated from two ~8-week-old calves with enteritis; out of these, 115 were ELISA positive and 78 were ELISA negative.

From six \textit{E. coli} strains isolated from children <6 months old, five were ELISA positive; these consisted of four \textit{O}26 strains and one \textit{O}111 strain, all of which contained the \textit{eae} and VT virulence factors, as did the one ELISA-negative \textit{O}111 strain. The results of the field survey of mixed cultures from human and animal diarrhea cases are summarized in Table 4. ELISA-positive reactions were obtained with 7.1 to 11.2% of the cultures tested from bovine, porcine, ovine, and human origins; single cultures were also recorded positive for the 8 canine and 31 avian strains examined. Pure ELISA-positive strains were obtained from 12 of these mixed cultures; these consisted of two \textit{O}8 strains, from human samples, and 10 \textit{O}26 strains, one from an ovine sample and nine from bovine samples. Only six of these (one \textit{O}8 and five \textit{O}26 strains) retained their ELISA-positive activity on retesting following storage at ~70°C. The single \textit{O}8 strain and three of the \textit{O}26 strains were PCR positive for both \textit{eae} and the gene encoding VT, and the other two \textit{O}26 strains were positive for only \textit{eae} or only the gene encoding VT. The six strains that had lost their ELISA activity were PCR negative for these virulence factors.

\textbf{Immunoblotting.} A strong immunoblot reaction was demonstrated at approximately 21 kDa for strain 4276 (\textit{O}26, \textit{eae} and VT positive), with MAb dilutions of up to 1:10,000 (Fig. 1). No immunoblot reactions were observed for K-12 or for strain 1045 (\textit{O}26, \textit{eae} and VT negative), S784 (\textit{O}117, \textit{eae} and VT positive), or 3680 (\textit{O}157, \textit{eae} positive and VT negative).

\section*{DISCUSSION}

The MAb 2F3 produced in this study demonstrated a high level of specificity for a group of \textit{E. coli} strains, in particular, strains of serotype \textit{O}26, with the potential to express the EHEC and EPEC virulence factors of \textit{eae} and VT. \textit{eae} is the gene for the expression of intimin, which is regarded as a significant virulence factor in both EHEC and EPEC strains. If it is assumed that all strains with \textit{eae} are potentially pathogenic, the application of MAb 2F3 in a sandwich ELISA format enables the rapid detection of a group of pathogenic strains from within these groups.

The identity of the antigen detected by MAb 2F3 is not clear. Immunoblotting demonstrated a strong reaction at 21 kDa with only the \textit{eae}- and VT-positive \textit{O}26 antigen used. From the molecular weight and surface presence of this protein, it is possible that it is intimab (14) or the recently described EspA protein (18). The former is indicated by the loss of antigen from six strains following storage, possibly from plasmid loss. Fimbriae implicated in early host cell adhesion of EPEC strains, named the bundle forming pili (\textit{bfp}), have been defined as plasmid located (14). Giron et al. (15), using a molecular probe, demonstrated that bfp were only present in the EPEC strains of the human pathogenic \textit{E. coli} strains that were examined. China et al. (9) failed to find bfp in animal EPEC or EHEC strains by using this human EPEC probe. Wieler et al. (36) demonstrated a significant increase in cell attachment of bovine EHEC \textit{O}118 strains on fetal calf lung cells (90.5%) compared with human HEp-2 cells (52.4%) by using the fluorescent actin staining test (17). These studies indicate differences in adhesins between EPEC and EHEC.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Strain origin & ELISA & Total no. of isolates & No. \textit{eae} positive & No. VT positive & No. \textit{eae} and VT positive \textit{eae} \textit{eae}
\hline
\textbf{O26} & + & 33 & 10 & 3 & 18 \textit{eae} \textit{eae} \\
& - & 10 & 1 & 0 & 10 \textit{eae} \textit{eae} \\
\textbf{O18} & + & 3 & 1 & 0 & 1 \textit{eae} \textit{eae} \\
& - & 11 & 0 & 0 & 11 \textit{eae} \textit{eae} \\
\textbf{O111} & + & 6 & 3 & 0 & 3 \textit{eae} \textit{eae} \\
& - & 2 & 0 & 2 & 0 \textit{eae} \textit{eae} \\
\textbf{O157} & + & 0 & 0 & 0 & 0 \textit{eae} \textit{eae} \\
& - & 10 & 1 & 9 & 0 \textit{eae} \textit{eae} \\
\textbf{Others} & + & 4 & 1 & 1 & 1 \textit{eae} \textit{eae} \\
& - & 9 & 2 & 5 & 2 \textit{eae} \textit{eae} \\
\hline
\end{tabular}
\caption{Characterization of the \textit{E. coli} strains demonstrating positive and negative reactions in the MAb 2F3 sandwich ELISA}
\end{table}
strains. It is possible that the antigen detected in the present study is an alternative to bfp for preliminary cell attachment. If this is confirmed, since it was demonstrated in both human- and animal-isolated strains, it must be concluded that either there is a common host receptor or that these strains possess more than a single host cell attachment mechanism.

The loss of the antigen detected by the ELISA in six strains which did not possess either the eae or the VT factor and its presence in a small number of VT-positive and eae-negative isolates demonstrate its occurrence in non-EPEC and non-EHEC strains. It can be speculated that the presence of eae and/or VT provides some plasmid stability, but whether these strains are of any pathological significance is unknown and requires further investigation. Nonpathogenic strains, such as those from the same VT-producing strains, can express a virulence factor(s).

It is recognized that virulence is the result of a combination of factors which, individually, have limited pathogenic effect. Apart from experimental infection, the significance of these factors in bacterial strains is determined by their presence in combination with other factors, and by their more-common occurrence in strains isolated from diseased animals or humans. The association of the vast majority of E. coli strains that reacted with MAb 2F3 to the presence of the gene for intimin, which is regarded as a virulence factor of notable significance, is a strong indication of the importance of the antigen to which it reacts.

**TABLE 4. Results obtained with the MAb 2F3 sandwich ELISA on field isolates from enteritis cases**

<table>
<thead>
<tr>
<th>Strain origin</th>
<th>No. tested</th>
<th>No. ELISA positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>366</td>
<td>41 (11.2)</td>
</tr>
<tr>
<td>Porcine</td>
<td>42</td>
<td>3 (7.1)</td>
</tr>
<tr>
<td>Ovine</td>
<td>40</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Canine</td>
<td>8</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Avian</td>
<td>31</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Human</td>
<td>490</td>
<td>44 (9.2)</td>
</tr>
<tr>
<td>Total</td>
<td>985</td>
<td>93 (9.4)</td>
</tr>
</tbody>
</table>

**FIG. 1.** Nitrocellulose immunoblot with MAb 2F3 with whole-cell preparations of E. coli strains K-12 (A), 1045 (O26; eae and VT negative) (B), 4276 (O26; eae and VT positive) (C), S784 (O157; eae and VT positive) (D), and 3680 (O157; eae positive and VT negative) (E).

The high prevalence of O26 strains amongst the positive reactants to this MAb indicates the probable significance of this serotype in animal enteritis in Northern Ireland. A number of O26 strains were also present in the ELISA-negative group, the majority of these being eae and VT negative. This indicates that other pathogroups of this serotype are of probable significance in this condition.

Although only a small number of strains from human diarrhoea were tested, the demonstration, by ELISA, of a common antigen in bovine and human isolates could indicate a zoonotic risk of bovine strains to humans. The commonality of the MAb-detected antigen was also demonstrated in the results obtained with isolates from the field survey of human and animal enteritis (Table 4). The fact that strains from the same serotypes have been implicated in both bovine and human diseases (O26, O111, and O18) also supports these findings. The presence of a high percentage of EHEC and EPEC strains in both ELISA-positive and -negative groups isolated from healthy calves sampled at an abattoir (Table 4) indicates a significant potential for infection of susceptible cattle and for zoonotic transfer to humans.

The results of the survey conducted with nearly 1,000 animal and human enteric isolates demonstrated a significant presence of strains expressing the targeted antigen (Table 4), in particular from bovine, human, ovine, and porcine samples. Although only five EHEC-EPEC strains were purified from these samples by the limited method employed, the presence of a virulence-associated antigen was demonstrated in a high proportion of the mixed cultures. This finding indicates a significant pathogenic role of EHEC and EPEC strains in both human and animal enteric diseases and highlights the diagnostic potential of the assay developed. Further studies to develop MAbs to surface antigens of the eae-positive strains that were nonreactive to the O26 MAbs in this study would clarify the significance of the antigens in terms of virulence and virulent-strain detection.

**ACKNOWLEDGMENTS**

The technical assistance of Neill Brice is gratefully acknowledged, as is the help of our colleagues from the institute’s diagnostic laboratory. In addition, we are indebted to Vinciane Pirson for the work carried out at Liège.

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