JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1991, p. 99–103
0095-1137/91/010099-05$02.00/0

Vol. 29, No. 1

Production and Characterization of a Monoclonal Antibody Specific for Enterohemorrhagic *Escherichia coli* of Serotypes O157:H7 and O26:H11

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Received 8 June 1990/Accepted 8 October 1990

A monoclonal antibody (MAb 4E8C12) specific for *Escherichia coli* O157:H7 and O26:H11 was produced by immunizing BALB/c mice with a rough strain of *E. coli* O157:H7. The antibody reacted strongly by a direct enzyme-linked immunosorbent assay with each of 36 strains of *E. coli* O157:H7. No cross-reactivity was observed with strains of *Salmonella* spp., *Yersinia enterocolitica*, *Shigella dysenteriae*, *Proteus* spp., *Escherichia hermanii*, *Klebsiella pneumoniae*, *Campylobacter jejuni*, *Serratia marcescens*, *Citrobacter* spp., *Enterobacter cloacae*, *Hafnia alvei*, *Aeromonas hydrophila*, and all except five strains of *E. coli* other than serotype O157:H7 (including strains of serotype O157 but not H7). The *E. coli* strains (all of serotype O26:H11) that reacted with the antibody were enterohemorrhagic *E. coli* (EHEC) that were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis and produced verotoxin similar to that of *E. coli* O157:H7. MAb 4E8C12 belongs to the subclass immunoglobulin G2a and has a kappa light chain. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of *E. coli* of different serotypes followed by Western immunoblot analysis revealed that MAb 4E8C12 reacted specifically with two proteins of EHEC strains of serotypes O157:H7 and O26:H11 with apparent molecular weights of 5,000 to 6,000. These proteins appeared to be markers specific for EHEC strains of serotypes O157:H7 and O26:H11. This MAb, because of its specificity, may be a useful reagent of an immunoassay for the rapid detection of these types of EHEC isolates in clinical and food specimens.

Escherichia coli O157:H7, the etiologic agent of hemorrhagic colitis (17, 22, 23) and a cause of hemolytic uremic syndrome (6, 7), is now recognized as an important cause of human morbidity and mortality. Most outbreaks of hemorrhagic colitis have been food related, with undercooked ground beef or raw milk principally identified as vehicles of infection (2, 8). Hence, there is need for a rapid, sensitive, and specific assay for detecting *E. coli* O157:H7. The available methods for detecting the organism in foods generally are extremely time-consuming or are not highly specific.

Immunoassays that have been developed for *E. coli* O157:H7 detection react with verotoxin(s) [VT(s)] or *E. coli* O157 antigen and cross-reacting somatic antigens of *Salmonella* group N, *Yersinia enterocolitica*, and *Brucella* spp. (3, 15, 21); hence, they are not highly specific. Gene probe-based assays with DNA that encodes VT(s) (16, 18), detect all VT type 1 (VT-1) and VT-2-producing *E. coli* strains rather than only enterohemorrhagic *E. coli* (EHEC) strains that include serotypes O157:H7 and O26:H11 (9, 10). Levine et al. (10) prepared a DNA probe based on the 60-MDa plasmid typically carried by *E. coli* O157:H7. They determined that this probe hybridized with most strains of EHEC. However, because plasmids of *E. coli* may be lost during isolation, this probe would not detect EHEC isolates that no longer carry the 60-MDa plasmid. The objective of this study was to produce a monoclonal antibody (MAb) that is highly specific for EHEC and that can be used in an immunoassay for the rapid detection of *E. coli* O157:H7 in clinical and food specimens.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 strain 932, *E. coli* HA1 (a rough strain derived from *E. coli* O157:H7 strain 932), 34 other strains of *E. coli* O157:H7, 5 strains of *E. coli* O26:H11, 37 *E. coli* strains other than serotype O157:H7 or O26:H11, 1 *Escherichia hermanii* strain, 12 *Yersinia enterocolitica* strains, 17 *Salmonella* sp. strains, 5 *Campylobacter jejuni* strains, 2 *Shigella dysenteriae* strains, 3 *Proteus* sp strains, 13 *Klebsiella pneumoniae* strains, 1 *Klebsiella oxytoca* strain, 5 *Citrobacter* sp. strains, 2 *Enterobacter cloacae* strains, 1 *Hafnia alvei* strain, 4 *Aeromonas hydrophila* strains, and 1 *Serratia marcesens* strain were used. All bacteria were grown in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 16 to 18 h with agitation (100 rpm).

VTs. VT-1 and VT-2 from *E. coli* O157:H7 strain 932 were purified by the procedures described by Padhye et al. (12, 13).

Immunization of mice. *E. coli* HA1 cells were grown in TSB at 37°C for 16 h with agitation (100 rpm). The cells were harvested by centrifugation (1,500 × *g* for 10 min), washed three times with 0.01 M phosphate-buffered saline (pH 7.2), and then treated with 2% Formalin for 1 week at 37°C. Ten BALB/c mice (male; age, 6 to 8 weeks) were immunized by intraperitoneal injection of 2×10^8 Formalin-treated cells. Thereafter, every 4 weeks the mice received intraperitoneally the same number of Formalin-treated *E. coli* HA1 cells until the sera obtained by periodic bleeding of mice had titers of greater than 1:400. This generally required three inoculations. Four days before cell fusion, the mice were given a final intravenous injection of 10^8 Formalin-treated cells. At 4 to 5 months after the initial injection, mice were sacrificed and their spleen cells were fused with myeloma cells.

Fusion and cloning. Fusion and cloning were performed by the procedure of Galfre (4), with minor modifications. Briefly, spleen cells from immunized mice were fused with Sp 2/O-Ag-14 myeloma cells by using 40% polyethylene glycol (molecular weight, 1,300 to 1,600) and were grown in selective medium containing hypoxanthine, aminopterin, and thymidine with 0.3% fresh mouse erythrocytes. Supernatants from wells with hybridoma growth were screened for the production of antibodies against *E. coli* O157:H7 by a direct enzyme-linked immunosorbent assay (ELISA) as described below. Hybridomas of interest were cloned twice by limiting dilution at 0.5 and 0.1 cell per well in a medium containing 20% fetal bovine serum (GIBCO, Grand Island, N.Y.) and were reassayed for antibody production.

Direct ELISA. Antibody production was determined by an ELISA performed in 96-well styrene enzyme immunoassay-radioimmunoassay plates (GIBCO). Each well was coated with 100 μl of bacterial cells (*E. coli* O157:H7 strain 932, *E. coli* O157:H16, *E. coli* O2:K1:H7, or *E. coli* K-12 [negative control]) (ca. 10^7 cells per ml, optical density [OD] at 640 nm of 0.5) in 50 mM carbonate buffer (pH 9.6) and was rotated overnight on an orbital shaker at room temperature. After the wells were washed four times with 50 mM Tris hydrochloride (pH 7.5) plus 150 mM NaCl (TBS), the remaining binding sites were blocked with 5% bovine serum albumin in TBS. After 1 h of incubation at 37°C, the blocking buffer was removed and 100 μl of MAb (hybridoma supernatant) was added to the wells. The plates were incubated at 37°C for 1 h, and then the wells were washed four times with TBS plus 0.05% Tween-20 (TBS-T). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; 100 μl per well; 1:1,400 in TBS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.) was added and incubated at 37°C for 1 h. After the wells were washed four times with TBS-T, 100 μl of 2,2'-azino-di-[3-ethylbenzthiazoline sulfate]–peroxidase substrate (Kirkegaard and Perry Laboratories Inc.) was added per well. After 15 min of incubation at room temperature, the reaction was stopped by adding 50 μl of 1% sodium dodecyl sulfate (SDS) in TBS per well. The OD at 410 nm of each well was measured with a Dynatech MR 600 microplate reader.

The reproducibility of the assay was determined by duplicate testing. The sensitivity of the MAb-based ELISA was determined by using different levels of eight strains of *E. coli* O157:H7. The specificity of the MAb was determined by using the ELISA procedure with many different members of the family *Enterobacteriaceae* or enteric bacteria (the genera and the numbers of strains evaluated are indicated above).

Ascitic fluid. Ten BALB/c mice were primed by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane; Sigma Chemical Co., St. Louis, Mo.). Ten days later, mice were injected with 2×10^6 hybridoma cells. Mouse ascitic fluid was collected from 10 through 20 days postinjection. Cell debris and fibrin clots were removed by centrifugation (8,000 × *g*, 10 min), and antibody-containing fluids were stored at −20°C.

Purification of MAb. MAb from ascitic fluid was purified by using a protein A column (Immunopure plus IgG purification kit; Pierce Chemical Co., Rockford, Ill.) by a modification of the instructions of the manufacturer. Briefly, ascitic fluid was centrifuged at 10,000 × *g* for 20 min, and IgG binding buffer (pH 8.0) was added to the supernatant (3:1). This solution (4 ml) was applied to the column, and the pure MAb was eluted with IgG elution buffer (pH 2.8). Fractions of 1 ml were collected, and protein levels were monitored by measuring the OD at 280 nm. Fractions with proteins were combined and were dialyzed against 20 mM phosphate buffer (pH 7.0) overnight at 4°C.

Protein concentration was determined by the procedure described by Smith et al. (20) by using Pierce bicinchoninic acid protein reagent (Pierce Chemical Co.) and bovine serum albumin as a standard. The activity of purified antibody was determined by direct ELISA, and purity was determined by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoglobulin isotyping. Immunoglobulin isotyping was done by ELISA by using class-specific antisera by the protocol described by Padhye et al. (13), with a modification. The protocol was modified by coating wells of enzyme immunoassay plates with *E. coli* O157:H7 strain 932 (10^7 cells per ml) in 50 mM carbonate buffer (pH 9.6).

Preparation of OMPs. Outer membrane proteins (OMPs) were isolated by the method described by Hancock and Naikaido (5), with minor modifications. *E. coli* O157:H7 strain 932, *E. coli* HA1, *E. coli* O157:H16, *E. coli* O157:H45, and *E. coli* O26:K60:H11 were grown individually in 2 liters of TSB at 37°C for 18 h with agitation (150 rpm). Cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), and subsequent operations were performed at 4°C. Cells were washed with 0.01 M phosphate buffer (pH 7.2) containing 170 mM NaCl (phosphate-buffered saline) and sedimented by centrifugation, and the pellets were resuspended in the same buffer to a calculated OD at 640 nm of 40. Cells were broken in a French press (1400 lb/in^2) (American Instrument Co., Silver Spring, Md.), and cell debris was removed by centrifugation (5,000 × *g*, 5 min). OMPs were sedimented from the supernatant by centrifugation at 200,000 × *g* for 1 h. Pellets were resuspended in 0.01 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) at an approximate protein concentration of 20 mg/ml. The protein solutions were layered onto a 35 to 55% (wt/vol) sucrose gradient prepared in 0.01 M HEPES buffer, and OMPs were pelleted by centrifugation at 131,000 × *g* for 36 h. The OMPs were resuspended in 0.01 M HEPES buffer containing 1 mM $MgCl_2$ and sedimented by centrifugation (200,000 × *g*, 1 h). Pellets were resuspended in the same buffer and stored at −20°C. Protein concentrations were measured by the procedure described above (20).

Immunoblotting. OMPs (2.5 to 10 μg) and purified VT-1 and VT-2 (3 to 5 μg) were separated into individual protein bands by tricine-SDS-PAGE (19). The gel system consisted of a 16.5% separating gel, a 10.0% spacer gel, and a stacking gel with each gel made with 32:1 acrylamide-bisacrylamide. The gels were run in a double-slab electrophoresis cell (Protean; Bio-Rad Laboratories, Richmond, Calif.) at a constant 100 V until bromophenol dye reached 1 cm from the bottom of the gel. After electrophoresis, OMPs were transferred to a polyvinyl difluoride membrane (Immobilon; Millipore, Bedford, Mass.) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (wt/vol) by using a transblot apparatus (Bio-Rad Laboratories) at 100 V for 3 h. The transferred protein bands were stained immunochemically as follows. Nonspecific binding sites were blocked by incubation with 5% bovine serum albumin in TBS for 1 h at 37°C. After the gel was rinsed with 1% bovine serum albumin in TBS, the polyvinyl difluoride membrane was incubated with MAb (ascitic fluid diluted 1:6,000 in TBS) for 1 h at 37°C. The membrane was washed three times with TBS plus TBS-T and was incubated with alkaline phosphatase-labeled goat anti-mouse IgG (diluted 1:2,000 in TBS) for 30 min at 37°C. The membrane was washed thoroughly with TBS-T plus 0.05% SDS and treated with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium phosphatase substrate

(Kirkegaard & Perry Laboratories Inc.) to detect protein bands. Low-range and mid-range protein molecular weight markers (Promega, Madison, Wis.) were run on each gel, and each gel was stained with silver stain before and after transfer, to document the transfer of proteins.

RESULTS

Antibody-producing hybridomas were obtained from two different fusions. A total of 429 culture supernatants were screened, and 14 hybridomas producing antibodies reacting with *E. coli* O157:H7 were detected in the initial screening. Only one hybridoma, designated 4E8C12, produced antibodies that reacted specifically with *E. coli* O157:H7 but not the other *E. coli* serotype O157 non-H7 or non-O157 but H7 strains, i.e., O157:H16 and O2:K1:H7, used in the initial screening.

Isotyping revealed that MAb 4E8C12 is of the IgG2a class and has a kappa light chain. Subsequent analysis of the purified antibody by SDS-PAGE with and without reducing conditions revealed the presence of two bands and one band, respectively. The molecular weights of the two bands obtained under reducing conditions corresponded to the molecular weights of the heavy and light chains of IgG2a, which is consistent with our isotyping determination.

MAb 4E8C12 produced from hybridomas in mouse ascites had a high titer (1:6,000) with *E. coli* O157:H7, and as little as 0.3 μg of pure antibody could successfully detect *E. coli* O157:H7 in a direct ELISA. The MAb was highly reactive with all 36 strains of *E. coli* O157:H7, as determined by ELISA, with an OD of >1.0 for all strains tested. The specificity of MAb 4E8C12 was determined by ELISA by using *E. coli* strains other than serotype O157:H7 and several strains of *Y. enterocolitica*, *Salmonella* sp., *E. cloacae*, *C. jejuni*, *S. dysenteriae*, *Proteus* sp., *A. hydrophila*, *H. alvei*, *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, and *Citrobacter* sp. Five strains other than serotype O157:H7, which were all *E. coli* O26:H11, also reacted with the MAb (Table 1). These strains were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis, and they produced VT identical to that produced by *E. coli* O157:H7 (1, 7, 11). Eleven other strains of VT-producing *E. coli* that were not serotypes O157:H7 or O26:H11 but that were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis did not react with MAb 4E8C12 (Table 1).

The cellular component of *E. coli* O157:H7 and O26:H11 that reacted with MAb 4E8C12 was determined by tricine-SDS-PAGE followed by Western immunoblot analysis. OMPs from *E. coli* O157:H7 strain 932, *E. coli* HA1, *E. coli* O157:H16, *E. coli* O157:H45, and *E. coli* O26:H11 were separated by a special tricine-SDS-PAGE procedure because of the necessity to separate low-molecular-weight proteins with high resolution (Fig. 1A). The separated proteins from a second gel were transferred to a polyvinyl difluoride membrane, and the location of antigen recognized by MAb 4E8C12 was determined by Western blot analysis. The MAb reacted with two 5,000- to 6,000-molecular-weight OMPs of *E. coli* O157:H7 strain 932, *E. coli* HA1, and *E. coli* O26:H11 (Fig. 1B). No cross-reaction was observed with either *E. coli* O157:H16 or *E. coli* O157:H45. In addition, purified VT-1 and VT-2 were tested for reactivity with MAb 4E8C12 by Western blot and direct ELISA. No cross-reactivity with either toxin was observed (data not shown).

The sensitivity (minimum number of *E. coli* O157:H7 cells detected) of MAb 4E8C12 in the direct ELISA was determined with eight different strains of *E. coli* O157:H7. The detection limit was in the range of 10^4 to 10^5 cells per ml (Fig. 2).

TABLE 1. Reactivities of MAb 4E8C12 with *E. coli* O157:H7 and other enteric bacteria

Organism	No. of strains	
	Tested	Positive[a]
Escherichia coli		
O157:H7 (VT^+)[b]	35	35
O157:H7 (VT^-)[c]	1	1
O157:H16 (VT^-)	1	0
O157:H19 (VT^-)	1	0
O157:H25 (VT^-)	1	0
O157:H45 (VT^-)	1	0
O157:H^- (VT^-)	1	0
O2:K1:H7 (VT^-)	1	0
O18:K1:H7 (VT^-)	1	0
O26:H11 (VT^+)[d]	5	5
VT-producing *E. coli*; not serotypes O157:H7 or O26:H11[e]	11	0
Non-VT-producing strains; not serotypes O157:H7 or O26:H11	19	0
Escherichia hermanii	1	0
Proteus spp.	3	0
Klebsiella pneumoniae	13	0
Klebsiella oxytoca	1	0
Citrobacter sp.	11	0
Serratia marcescens	1	0
Shigella dysenteriae	2	0
Salmonella sp.[f]	17	0
Campylobacter jejuni	5	0
Yersinia enterocolitica	12	0
Enterobacter cloacae	2	0
Hafnia alvei	1	0
Aeromonas hydrophila	4	0

[a] OD at 410 nm of 0.2 above the background was considered positive; all positive strains had ODs of >1.0 above background.
[b] Two strains were cured of the 60-MDa plasmid; VT^+, VT was produced.
[c] VT^-, VT was not produced.
[d] *E. coli* O26:H11 strains were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis.
[e] VT-producing *E. coli* strains were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis; these strains included serotypes O4:NM, O5:NM, O45:H2, O50:H7, O111:H8; O111:NM, O113:H21, O125:NM, O145:NM, and O172:NM.
[f] Includes *Salmonella urbana* (group N).

DISCUSSION

MAbs that react with *E. coli* O157:H7 have been reported previously (14). These MAbs bind to the somatic O157 antigen; hence, they react with all *E. coli* strains belonging to serogroup O157, and they also cross-react with group N salmonellae (21). In contrast, the MAb described in this paper did not react with the O157 antigen and was, with the exception of one other serotype of *E. coli*, very specific for *E. coli* O157:H7. The MAb also reacted with five strains of *E. coli* O26:H11 which, like *E. coli* O157:H7, are EHEC that produce VT and cause hemolytic uremic syndrome and hemorrhagic colitis (1, 6, 9, 10). Interestingly, MAb 4E8C12 did not react with any of 11 other VT-producing *E. coli* of serotypes other than O157:H7 or O26:H11 that were isolated from patients with hemolytic uremic syndrome or hemorrhagic colitis. Apparently, the 5,000- to 6,000-molecular-weight OMPs of *E. coli* O157:H7 and O26:H11 that reacted with MAb 4E8C12 are unique to these types of EHEC isolates. These OMPs appear to be chromosomally encoded

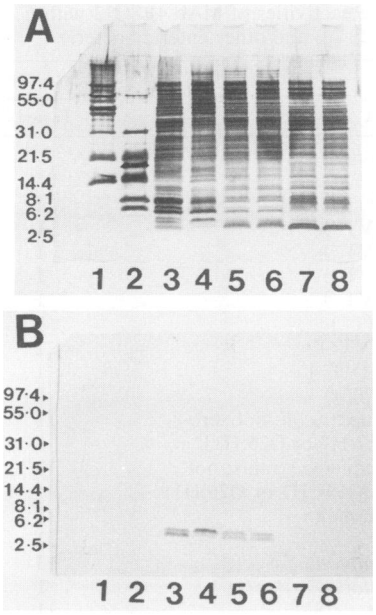

FIG. 1. (A) Tricine-SDS-PAGE profile of OMPs of *E. coli* O157:H7 strain 932 (lane 3), *E. coli* HA1 (lane 4), *E. coli* O26:H11 strain 84-381 (lane 5), *E. coli* O26:H11 strain 89-326 (lane 6), *E. coli* O157:H16 (lane 7), and *E. coli* O157:H45 (lane 8). A 5-μg sample of OMP was applied per lane. Low-molecular-weight standards (indicated on the left, in thousands) are shown in lanes 1 and 2. The gel was silver stained. (B) Western blot of SDS-PAGE-separated OMPs treated with MAb 4E8C12. Lane numbers correspond with the OMP preparations indicated in panel A.

because strains of *E. coli* O157:H7 cured of the 60-MDa plasmid react with MAb 4E8C12 in direct ELISA. The detection limit with this MAb in the direct ELISA was 10^4 to 10^5 *E. coli* O157:H7 cells per ml; however, we are attempting to enhance the expression of the 5,000- to 6,000-molecular-weight OMP recognized by MAb so that the sensitivity of an immunoassay can be increased. Properties of these unique OMPs will be described in another report.

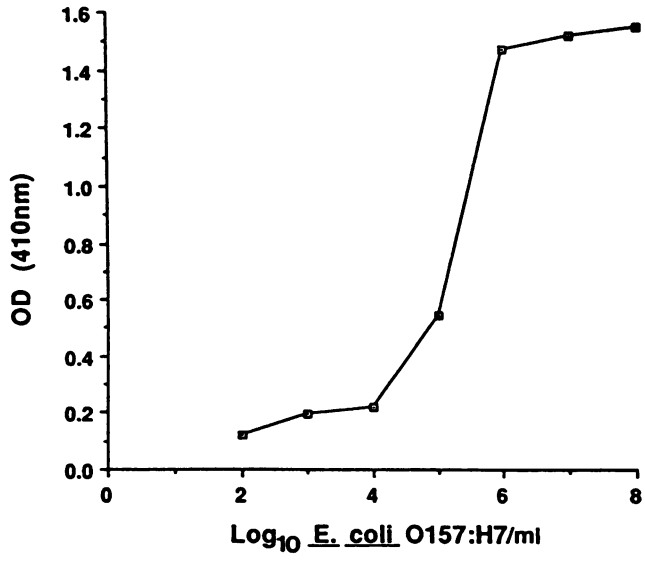

FIG. 2. Sensitivity of the direct ELISA technique for detecting *E. coli* O157:H7 strain 932.

MAb 4E8C12 was obtained by immunizing BALB/c mice with a rough strain of *E. coli* O157:H7 which lacks a portion of the lipopolysaccharide (LPS) layer of the cell outer membrane. This strain (HA1) was obtained while attempting to isolate a variant of *E. coli* O157:H7 strain 932 that was highly adherent to Henle 407 cell monolayers. Strain 932 was passaged several times on Henle 407 monolayers. Each time only those bacteria that remained attached to the monolayers after washing were cultured. Strain HA1, which grows as rough colonies on culture media, was isolated from passage 11. One reason for selecting a rough strain was because of previous difficulties encountered in obtaining a MAb specific for *E. coli* O157:H7. Previous studies with whole cells of a smooth (complete LPS layer) strain of *E. coli* O157:H7 as an immunogen produced MAbs that reacted principally with O157 somatic antigen, which is the outer component of the LPS layer. These MAbs also cross-reacted with many strains of *Y. enterocolitica*.

The LPS layer of the cell envelope is a major surface antigen of gram-negative bacteria that is immunodominant; hence, most antibodies raised against whole cells react with a constituent of LPS. Perry et al. (15) have shown that the serological cross-reactions which occur between *E. coli* O157:H7 and other bacterial species such as *Y. enterocolitica* serotype O:9, *Salmonella* group N (O:30), and *Brucella abortus* are related to the presence of 1→2 linked 4-amino-4,6-dideoxy-α-D-mannopyranose residues in the *O*-polysaccharide repeating unit of their LPS. Hence, the presence of LPS in the immunizing antigen often yields LPS-directed antibodies that are not highly specific for *E. coli* O157:H7.

While investigating a food-borne outbreak caused by *E. coli* O157:H7, we isolated several strains of *K. pneumoniae*, *Citrobacter* sp., *Enterobacter* sp., *H. alvei*, and *A. hydrophila* from suspect food which gave false-positive reactions by a commercial *E. coli* O157 latex agglutination test. MAb 4E8C12 did not react with any of the bacterial strains that gave false-positive reactions by the commercial latex agglutination procedure, further indicating the specificity of the MAb.

MAb 4E8C12 is unique from other reported MAbs to *E. coli* O157:H7 because of its specificity for EHEC strains of serotypes O157:H7 and O26:H11. ELISA results indicate that the MAb has the required specificity, affinity, and sensitivity for detection of all *E. coli* O157:H7 and O26:H11 strains. Hence, this antibody can serve as a useful reagent in immunoassays to detect *E. coli* O157:H7 and O26:H11 in clinical and food specimens. Studies are under way to develop a rapid test for detecting *E. coli* O157:H7 by using this MAb.

ACKNOWLEDGMENTS

We thank Alan Junkins for providing strain HA1. We thank Herme Lior, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada; I. Kaye Wachsmuth and Nancy Strockbine, Centers for Disease Control, Atlanta, Ga.; and M. Karmali, The Hospital for Sick Children, Toronto, Ontario, Canada, for providing bacterial cultures and Zhao Tong for valuable assistance.

This work was supported in part by grants from the Wisconsin Milk Marketing Board and the National Livestock and Meat Board, by the College of Agricultural and Life Sciences, University of Wisconsin—Madison, and by contributions to the Food Research Institute.

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