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Antibodies to intimin and *Escherichia coli* secreted proteins A and B in patients with enterohemorrhagic *Escherichia coli* infections

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Abstract Enterohemorrhagic *Escherichia coli* produce an attaching and effacing lesion upon adhering to the intestinal epithelium. Bacterial factors involved in this histopathology include the intimin adhesin and *E. coli* secreted proteins (Esps) A and B. In this study we investigated the serum antibody responses to recombinant *E. coli* O157:H7 intimin, EspA, and EspB by immunoblotting. Canadian patients with O157:H7 infection (*n*=10), Swedish patients with O157:H7 (*n*=21), non-O157 (*n*=18), or infection from which the serotype was not available (*n*=3), and asymptomatic household members (*n*=25) were studied and compared with Canadian (*n*=20) and Swedish controls (*n*=52). In Canadian patients, IgG antibodies to intimin, EspA, and EspB were analyzed, in Swedish patients and their household members IgA, IgG, and IgM antibodies to EspA and EspB were studied. Patients and household members mounted an antibody response to the antigens. Significantly more patients developed an acute response to EspB compared with controls (P<0.01 Canadian patients, P<0.0001 Swedish patients). EspB IgA, IgG, and IgM had a specificity of 100%, 86%, and 86%, positive predictive value of 100%, 83%, and 81%, and sensitivity of 57%, 69%, and 63%, respectively, and appear to be an appropriate assay for the detection of EHEC infection. In cases of hemolytic uremic syndrome or hemorrhagic colitis this assay may be useful when a fecal strain has not been isolated, or in epidemics of non-O157 infection.

Keywords Enterohemorrhagic *Escherichia coli* · Intimin · *Escherichia coli* secreted protein A · *Escherichia coli* secreted protein B · Hemolytic uremic syndrome · Hemorrhagic colitis

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are causal agents of diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) that have been associated with large outbreaks related to contaminated food and water [1]. EHEC express two factors strongly associated with virulence, Shiga toxins (Stx) and intimin, as well as a number of potential virulence factors, such as *E. coli* secreted proteins (Esps), lipopolysaccharide, and enterohemolysin [2]. Many serotypes of EHEC have been reported, of which the most prevalent is *E. coli* O157:H7 [3].

The diagnosis of EHEC infection is based on the isolation of the bacterial strain from the feces of infected individuals, the identification of specific virulence factors in the feces of patients by phenotypic (free fecal toxin) or genotypic (polymerase chain reaction for *stx*) detection assays, or by serological assays that detect antibodies to the lipopolysaccharide of the strain. The latter assays are available for a number of EHEC serotypes, such as *E. coli* O157, O26, O55, O103, O111, and O128 [4, 5, 6, 7, 8].

Detection of EHEC in infected patients may be limited by the insensitivity of fecal cultures employing sorbitol-MacConkey agar, and hampered if the strain is no longer

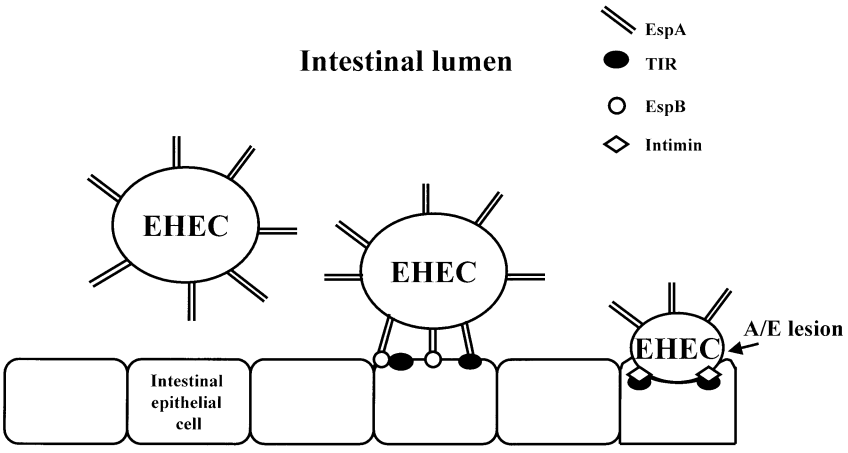

Fig. 1 Diagram of the formation of the attaching and effacing (*A/E*) lesion on the intestinal epithelium. On the left, enterohemorrhagic *Escherichia coli* (EHEC) is seen in the intestinal lumen before adherence to the epithelium. Esp A is a filamentous organelle protruding from the bacteria. In the middle of the figure EHEC attach to the intestinal cell during which Tir, Esp B, and Esp D are injected into the cell via EspA allowing intimate adherence between intimin and Tir as seen on the right. EspB and EspD are required for signal transduction events occurring within the cell during the formation of the A/E lesion

present in the intestinal flora due to antibiotic treatment or if the fecal culture is taken long after the onset of symptoms. Serological detection enables the identification of a limited number of non-O157 strains. However, over 200 serotypes of non-O157 strains have been reported [9], and over 100 of these serotypes have been isolated from symptomatic humans [10, 11]. Therefore, a serological diagnostic method, which would permit detection of EHEC infection regardless of serotype, is desirable.

Like enteropathogenic *E. coli* (EPEC), EHEC produce an attaching and effacing (A/E) lesion upon adhesion to epithelial cells in vivo and in vitro. This lesion is characterized by intimate bacterial attachment and effacement of intestinal microvilli [12, 13]. The bacterial antigens required for this lesion are encoded on a chromosomal locus called the LEE (locus of enterocyte effacement), which includes the *eae* gene encoding the outer membrane protein intimin [14], genes encoding proteins secreted by a type III secretion system [15] entitled Esps (*E. coli* secreted proteins) and Tir (translocated intimin receptor, also termed EspE in EHEC O26:H–) [16, 17], and the *esc* and *sep* genes encoding the type III secretion appartus. The 94- to 97-kilodalton (kDa) protein intimin mediates intimate attachment of the bacterium [14, 18] and is thus required but not sufficient for the formation of the A/E lesion. EspA is a 25-kDa polypeptide that has been shown to form a filamentous organelle required for the translocation of EspB and Tir into the eukaryotic cell [19, 20]. EspB (37 kDa) and EspD (39 kDa) are polypeptides necessary for signal transduction events occurring in the epithelial cell during the formation of the attaching and effacing lesion. They may form a pore in the host cell membrane through which bacterial proteins are injected into the eukaryotic cell [19, 21, 22, 23, 24]. Tir/EspE (80 kDa) is a bacterial protein that is translocated into the mammalian cell and serves as a cell receptor for intimin [16, 17]. Figure 1 shows a model of the formation of the A/E lesion on intestinal epithelium.

Most human isolates of Stx-producing *E. coli* possess the genes required for the formation of A/E lesions, although a few isolates that do not possess these genes have been described [25, 26, 27]. In previous studies, patients with EHEC O157:H7 or O111:H$^-$ associated HC and HUS were found to have antibodies against Tir, intimin, EspA, and EspB [15, 28, 29, 30, 31].

The aim of this study was to examine the antibody responses to recombinant intimin, EspA, and EspB (from *E. coli* O157:H7) in patients with EHEC-associated diarrhea and HUS regardless of serotype. Furthermore, the antibody response in asymptomatic household members was studied. The response profile was compared with that of anti-O157 lipopolysaccharide and anti-Stx, in order to evaluate the utility of EHEC antibodies for the diagnosis of this infection.

Material and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. EHEC O157:H7 wildtype strain 93111 was isolated from an outbreak of HC and HUS in Washington State in 1993 [32]. The His-intimin fusion protein was obtained from *E. coli* strain MG15(pREP4,pEB313) [30]. The cloning vector used to construct the His-EspA and His-EspB fusion proteins was obtained from Qiagen (Valencia, Calif., USA) in *E. coli* JM109(pQE30). *E. coli* K-12 strain DH5α(pREP4) (Bethesda Research Laboratories, Gaithersburg, Md., USA; Qiagen) was used as the host to express His-EspA and His-EspB fusion proteins. Bacterial strains were grown in Luria broth supplemented with ampicillin and/or kanamycin where appropriate.

Expression and purification of His-intimin, His-EspA, and His-EspB fusion proteins

The His-intimin fusion protein was purified from *E. coli* MG15 (pREP4,pEB313) by nickel affinity chromatography as previously described [30]. Following this, the protein was run on a 10% polyacrylamide gel under reducing conditions and visualized with a

Table 1 Bacterial strains and plasmids used in this study

E. coli strains/plasmids	Serotype and properties	Source (reference)
Strains		
93111	Wild-type *E. coli* O157:H7	P. Tarr [32]
MG15	Expression host	Qiagen
DH5α	Expression host	BRL
JM109	Cloning host	Qiagen
Plasmids		
pQE30	Histidine fusion cloning vector	Qiagen
pEB313	6XHis::*eaeA*	[30]
pCVD468	6XHis::*espA*	This study
pCVD469	6XHis::*espB*	This study
pREP4	*lacI*^{q}	Qiagen

BRL, Bethesda Research Laboratories, Life Technologies

copper stain (Bio-Rad, Hercules, Calif., USA). Due to the presence of several breakdown products, the band containing the full-length intimin protein (94 kDa) was excised from the gel, destained, and the intimin protein was electroeluted from the gel slice using the Model 422 Electro-eluter (Bio-Rad), according to the manufacturer's instructions. Protein was concentrated using an Amicon concentrator (Beverly, Mass., USA).

To construct plasmids expressing EspA and EspB, DNA segments encoding these proteins were amplified from strain 93111 as the template (for both constructs forward primers included a *Bam*HI site and reverse primers included a *Kpn*I site); EspA forward primer: 5'-CGCGGATCCGATACATCAAATGCAACATCCGT-3', reverse primer: 5'-CGGGGTACCGGTTATTTACCAAGGGATATT-3', EspB forward primer: 5'-CGCGGATCCAATACTATTGATAATACT-3', reverse primer: 5'-CGTGGTACCCCCAGCTAAGCGACCCGATTGC-3'. Segments were cloned respectively into a *Bam*HI-*Kpn*I multicloning site in plasmid vector pQE30, resulting in pCVD468 (His-EspA) and pCVD469 (His-EspB). Insertion of the cloned *espA* and *espB* genes was confirmed by sequencing [33]. The His-tagged proteins were purified according to the manufacturer's directions. Briefly, *E. coli* DH5α(pREP4,pCVD468) was grown overnight at 30°C and *E. coli* DH5α(pREP4,pCVD469) at 37°C in 5 ml Luria broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. These cultures were used separately to inoculate 100 ml of Luria broth under similar conditions and grown to an OD_{600} of 0.7, at which point *E. coli* DH5α(pREP4,pCVD468) was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Fisher Scientific, Fairlawn, N.J., USA) for 1 h and *E. coli* DH5α (pREP4,pCVD469) for 2 h, with vigorous shaking. The cells were harvested by centrifugation and the pellet frozen at –20°C. After sonication of the bacteria the proteins were denatured with urea, purified over Ni-NTA (nickel-nitrilotriacetic acid agarose) resin (Qiagen), dialyzed against 0.01 M phosphate-buffered saline (PBS), pH 7.4, separated by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Coomassie blue (Merck, Darmstadt Germany). The size of the purified proteins, EspA, 25 kDa, and EspB, 37 kDa, was consistent with the known molecular mass for these proteins [15].

Protein concentrations were determined by the Bio-Rad DC colorimetric assay (Bio-Rad) and by BCA Protein Assay Reagent (Pierce). Intimin was kept at –80°C and EspA and EspB at –20°C prior to use.

Preparation of bacterial lysates and secreted proteins from wild-type EHEC

Whole cell lysates from *E. coli* O157:H7 strain 93111 were prepared by growing the strain overnight in Luria broth at 37°C followed by shaking a 2-ml culture at 37°C to an OD_{600} of 0.8. Bacteria were pelleted by centrifugation (10,000 *g*, 10 min), and run on a SDS-14% PAGE gel. A protein of approximately 94 kDa, corresponding to the molecular mass of intimin, was identified by immunoblotting as described below. *E. coli* secreted proteins were prepared from *E. coli* 93111 culture supernatant as previously described [15].

Sera from Canadian patients and controls

Sera were analyzed from 10 children who were diagnosed with *E. coli* O157:H7 infection at The Hospital for Sick Children, Toronto. These were sporadic cases of infection. Six patients suffered from the HUS, 3 from bloody diarrhea, and 1 from non-bloody diarrhea. The children were aged 2–9 years (median age 3 years). Sera were taken upon admission and at follow-up 1–20 months (median 2 months) after admission. The sera were collected for various clinical indications. Unused portions of the sera were banked by the microbiology laboratory and analyzed for serological responses in this study.

Control sera consisted of banked sera from 20 children aged 1–9 years (median age 5 years) investigated for viral respiratory illnesses. These samples were also unused portions of sera submitted for routine tests. None of the pediatric controls had an admission diagnosis listed on the laboratory requisition form as diarrhea or HUS. All sera were stored at –20°C until assayed. The ethics committee of The Hospital for Sick Children, Toronto has approved anonymous analysis of sera for research purposes.

Sera from Swedish patients, their household members, and controls

Sera were analyzed from 42 patients diagnosed with EHEC-related disease at the Department of Pediatrics and the Department of Infectious Diseases, Lund University Hospital. All were sporadic cases of infection. Thirty-four patients suffered from HUS, 8 patients had HC. Of these patients there were 30 children with acute HUS (12 male and 18 female, aged 1–10 years, median age 3.7 years), from whom sera were taken upon admission (3–6 days after the onset of diarrhea). Twenty-eight of these patients had bloody diarrhea and 25 required dialysis. Convalescent sera were available from 17 children with HUS at follow-up 2–8 months (median 4 months) after admission (n=15) and 10–11 years after recovery (n=2). In 4 cases only convalescent, but not acute, sera were available; 2 of these patients had bloody diarrhea and the other 2 had non-bloody diarrhea. HUS was associated with *E. coli* O157:H7 (n=16), non-O157 (n=15), or not serotyped (n=3). Sera were taken from 25 asymptomatic household members of the HUS patients, 21 were parents (9 male and 12 female), and 4 were siblings (<18 years of age, 1 male and 3 female). These samples were obtained during the 1st week of the patients' hospitalization. Sera were also analyzed from 8 patients with acute HC (3 adults and 5 children aged 1–18 years, median 8 years), associated with *E. coli* O157:H7 (n=5) or non-O157 (n=3). Convalescent sera were available from 1 adult and 1 child after HC. Control sera were collected from 35 children (13 males and 22 females, aged 1–12 years, median 5 years) and 17 healthy adult volunteers. The pediatric controls did not have a history of recent diarrhea or HUS and were seen for follow-up of pyelonephritis, vasculitis, renal failure, migraine, renal agenesis, nephrotic syndrome, diabetes mellitus, epilepsy, anal atresia, and thrombotic thrombocytopenic purpura (TTP). The patients in the latter category were brothers

Table 2 Tests carried out in the Canadian and Swedish groups (*LPS* lipopolysaccharide)

Subjects	*n*	Test					
		Fecal strain genotype (*stx*, *eae*)	Antibodies to LPS	Antibodies to Stx	Antibodies to intimin	Antibodies to EspA	Antibodies to EspB
Canadian patients	10	10[a]	10	10	10[b]	10[b]	10[b]
Canadian controls	20	0	20	20	20[b]	20[b]	20[b]
Swedish patients	42	36	6	0	0	42[c]	42[c]
Swedish household members	25	23	0	0	0	25[c]	25[c]
Swedish controls	52	2[d]	2[d]	0	0	52[c]	52[c]

[a] Represents the number of subjects whose samples were tested
[b] In Canadian subjects IgG antibodies to intimin, EspA and EspB were assayed
[c] In Swedish subjects IgA, IgM, and IgG antibodies to EspA and EspB were assayed
[d] Pediatric controls with thrombotic thrombocytopenic purpura

with recurrent TTP [34]. Samples were taken with the informed consent of parents and stored at –20°C until assayed. The study was approved by the ethics committee of the University of Lund.

Identification of bacterial serotypes by analysis of patient stools or sera

A summary of the tests carried out on samples from feces and serum is shown in Table 2. Fecal samples from the Canadian patients (*n*=10) were obtained shortly after admission and examined for the presence of Stx-producing *E. coli*. *E. coli* O157:H7 were isolated by culture on sorbitol-MacConkey agar, serotyped, and toxin production was assayed by Vero cell cytotoxicity assay as previously described [35, 36]. *E. coli* O157:H7 were isolated from the feces of all the Canadian patients. Nine strains produced both Stx1 and Stx2, one strain produced only Stx1. All strains were found to be positive for the *eae* gene by polymerase chain reaction (PCR) as previously described [37].

Fecal samples were available from the 36 of 42 Swedish patients upon admission. Feces tested positively for the presence of the EHEC virulence genes *eae* and *stx* by PCR as previously described [38]. Thirty-four strains produced Stx2 and two strains produced Stx1 (Table 3). A fecal *eae*- and *stx*-positive strain was isolated in 33 patients and found to be *E. coli* O157 in 19 patients and non-O157 serogroups in 14 patients, which included O145 (*n*=3), O26 (*n*=1), O121 (*n*=3), O103 (*n*=2), O8 (*n*=1), and O non-typeable (*n*=4). In 6 patients a fecal strain was not available and sera taken upon admission were tested by ELISA for the presence of antibodies to the lipopolysaccharide of *E. coli* O157 as previously reported [39]. Sera from 2 of these patients were further tested for antibodies to *E. coli* O121, O26, O103, O111, and O145. The patients were found to have antibodies to O157 (*n*=2) and O121 (*n*=1) [40]; 3 patients did not have antibodies and were considered to have had a non-O157 infection (Table 3 patients 7, 8, 18). In 3 patients the identity of a bacterial strain as O157 or non-O157 could not be made; 2 of these patients' samples were PCR positive for EHEC *eae* and *stx* (patients 5, 11) and in the third patient feces was not available (patient 34 Table 3). Fecal samples were available from 23 of 25 asymptomatic household members from whom serum was analyzed. PCR testing of fecal samples was positive for the *eae* gene in 5 of these individuals; the same samples were positive for the *stx2* gene and 1 also tested positively for the s*tx1* gene. Fecal samples were obtained from the 2 pediatric controls with TTP. They tested negatively for the *eae* and *stx* genes. Serum samples from these 2 patients did not contain antibodies to O157 lipopolysaccharide.

Antibody responses to EHEC antigens: intimin, EspA, and EspB

Serum antibody responses to intimin, EspA, and EspB were assayed by immunoblotting. For the Canadian sera, immunoblots were carried out at the Center for Vaccine Development. Purified intimin, EspA, and EspB were run in the same lane on a SDS-14% PAGE gel; 500 ng of each protein in 5 µl PBS was placed in each lane in a Hoefer minigel apparatus (Pharmacia Biotech, Piscataway, N.J., USA). In a similar manner wild-type EHEC antigens, i.e., bacterial whole cell lysates and secreted proteins, were run in separate lanes on a gel. Separated proteins were electroblotted for 1 h onto Immobilon-P membranes (Millipore, Bedford, Mass., USA) using a Bio-Rad solid plate tank. Unreacted sites on the membrane were blocked for 1 h in bovine serum albumin (BSA, 5%, Sigma, St. Louis, Mo., USA) and 0.05% Tween 20 in PBS.

Membrane strips were incubated with human sera diluted 1:5,000 in PBS containing 1:10 of the blocking buffer. Three dilutions of human sera were investigated: 1:1,000, 1:5,000, and 1:10,000. The first gave extremely strong signals in positive sera, and the latter gave weak signals. A dilution of 1:5,000 was therefore chosen. Additional strips were incubated separately with polyclonal rabbit antibodies against EPEC antigens intimin (1:1,000) [41], EspA (1:5,000) [33], EspB (1:5,000) [42], or monoclonal antibody against the histidine tag (1:1,000) (Qiagen) for 1 h at room temperature. Similarly, lanes containing EHEC wild-type antigens were incubated with rabbit anti-intimin antibody [41] for the whole cell lysates and rabbit antiserum against all EPEC secreted proteins [15] for the EHEC secreted proteins (both at 1:1,000). After washing, the membranes were incubated for 1 h with a goat anti-human IgG horseradish peroxidase (HRP) conjugated antibody diluted 1:45,000, goat anti-rabbit IgG (1:30,000, both antibodies from Kierkegaard and Perry Laboratories, Gaithersburg, Md., USA), or anti-mouse IgG (1:35,000, Sigma-Aldrich, St. Louis, Mo., USA), as required. Bound immunoglobulin was detected by ECL chemiluminescence kit (Amersham Life Science, Piscataway, N.J., USA). Exposure time was approximately 1–2 min. Longer exposure times (up to 1 h) did not improve detection. All patient and control samples were coded and exposed for an equal length of time. Sera identity was decoded after film exposure.

Strips containing purified intimin, EspA, and EspB reacted with the appropriate antibodies, including the antibody against the histidine tag. Strips containing wild-type proteins reacted with rabbit anti-intimin antibody and rabbit antiserum against all EPEC secreted proteins.

For the Swedish sera, immunoblots were carried out at the Department of Pediatrics, Lund University. In order to achieve comparable results to those carried out at the Center for Vaccine Development, EspA and EspB concentrations of 250, 500, 1,000, 1,500, and 2,000 ng protein/lane were first tested by immunoblotting. A clear band was identified when concentrations of 500 ng or more were used. Positive samples became stronger but negative samples did not become positive when the concentration was increased. Purified EspA and EspB, 800 ng/protein, were run in the same lane on a SDS-14% PAGE gel in a Bio-Rad gel cell. The gels were electroblotted for 1 h onto a 0.45-µm Protran nitrocellulose transfer membranes (Schleicher and Schuell, Dassel, Germany) using a semi-dry electroblotter (Ancos, Denmark). Following transfer, membrane strips were blocked and incubated with human sera

Table 3 Criteria for diagnosis of enterohemorrhagic *E. coli* (EHEC) infection in Swedish patients (*HUS* hemolytic uremic syndrome, *HC* hemorrhagic colitis, *PCR* polymerase chain reaction, *na* not available, *ni* not isolated, *nt* not tested)

Patient number	Diagnosis HUS or HC	Diarrhea (D) or bloody diarrhea (BD)	stx gene (PCR) in feces	O serotype of fecal *E. coli* strain	Serum antibodies to EHEC LPS
1	HUS	BD	*stx2*	O145	nt
2	HUS	BD	*stx2*	O non-type	nt
3	HUS	BD	*stx2*	O26	nt
4	HUS	D	*stx2*	O145	nt
5	HUS	D	*stx2*	ni	nt
6	HUS	BD	*stx2*	O121	nt
7	HUS[a]	BD	nt	na	Negative for O157
8	HUS	BD	*stx2*	ni	Negative for O157
9	HUS	BD	*stx2*	O157	nt
10	HUS	BD	*stx2*	O157	nt
11	HUS	BD	*stx2*	ni	nt
12	HUS	BD	*stx2*	O157	nt
13	HUS	BD	*stx2*	O157	nt
14	HUS	BD	*stx2*	O121	nt
15	HUS	BD	nt	na	O157
16	HUS	BD	nt	na	O157
17	HUS	BD	nt	na	O121
18	HUS[a]	BD	nt	na	Negative for O157
19	HUS	BD	*stx2*	O157	nt
20	HUS	BD	*stx2*	O157	nt
21	HUS	BD	*stx2*	O157	nt
22	HUS	BD	*stx2*	O157	nt
23	HUS	BD	*stx2*	O157	nt
24	HUS	D	*stx2*	O157	nt
25	HUS	BD	*stx2*	O non-type	nt
26	HUS	BD	*stx2*	O157	nt
27	HUS	BD	*stx2*	O non-type	nt
28	HUS	BD	*stx2*	O non-type	nt
29	HUS	BD	*stx2*	O157	nt
30	HUS	BD	*stx2*	O145	nt
31	HUS	BD	*stx2*	O157	nt
32	HUS	BD	*stx2*	O157	nt
33	HUS	D	*stx1*	O103	nt
34	HUS[a]	D	nt	na	nt
35	HC	BD	*stx1*	O103	nt
36	HC	BD	*stx2*	O157	nt
37	HC	BD	*stx2*	O157	nt
38	HC	BD	*stx2*	O157	nt
39	HC	BD	*stx2*	O157	nt
40	HC	BD	*stx2*	O157	nt
41	HC	BD	*stx2*	O121	nt
42	HC	BD	*stx2*	O8	nt

[a] In these patients EHEC infection was not detected but assumed due to a diarrheal prodrome

as described for the Canadian sera. After washing, they were further incubated for 1 h with HRP-conjugated anti-human IgA 1:20,000, IgG 1:40,000, and IgM 1:5,000 (all antibodies from Dako, Glostrup, Denmark). A dilution of 1:40,000 was initially used for all three conjugated antibodies, but dilutions were decreased for anti-IgM-HRP and anti-IgA-HRP until detection was optimized. Bound immunoglobulin was detected by chemiluminescence. All patient and control samples were coded and exposed for an equal length of time (1–8 min). Detection was not improved by exposure of up to 15 min. Sera identity was decoded after film exposure.

Antibody responses to the lipopolysaccharide of *E. coli* O157:H7

Antibody responses to the lipopolysaccharide of *E. coli* O157:H7 were analyzed by ELISA in Canadian sera (diluted 1:320) taken upon admission and at follow-up, and in pediatric controls as previously described [43]. A value above a cut-off of 0.59 was considered positive.

Antibodies to Stx1 and Stx2

Antibodies to Stx were assayed in sera taken from Canadian patients upon admission and at follow-up and in pediatric control sera (diluted 1:100) by immunoblotting as previously described [44, 45].

Statistics

Differences between patients and controls with regard to immunoblot reactivity were evaluated with Fisher's exact test. Differences between these groups with regard to *E. coli* O157 lipopolysaccharide antibody results were evaluated by the Mann-Whitney test. $P<0.05$ was considered significant. The different serodiagnostic assays were compared with regard to sensitivity, specificity, and positive and negative predictive value [46].

Table 4 Antibodies to EHEC antigens in Canadian patients and controls

Patient group	Total number	Intimin IgG	EspA IgG	EspB IgG
HUS acute	6	5	5	4
HC acute	4	3	3	3
Total acute samples	10	8	8	7*
HUS convalescent	6	4	6	3
HC convalescent	4	4	4	3
Total convalescent samples	10	8	10	6*
Controls	20	7	9	2

*$P<0.01$ compared with controls; for statistical purposes HUS and HC patients (acute or convalescent) were combined and compared with controls

Results

Antibody responses to EHEC antigens in Canadian patients

Serum samples taken from each patient infected with *E. coli* O157:H7 during the acute phase of the disease and at convalescence were reacted by immunoblotting with recombinant intimin, EspA, and EspB. The antibody response to these proteins in patients and controls is summarized in Table 4. The acute and convalescent response of 1 patient is shown in Fig. 2. The sensitivity of the assay for detection of intimin antibodies during the acute phase of the disease was 80%, for EspA 80%, and for EspB 70%. The specificity was 65%, 55%, and 90% for intimin, EspA, and EspB, respectively. The positive predictive value was 53%, 47%, and 77% and negative predictive value was 87%, 85%, and 86%, respectively.

Patient and control sera (5 samples from each) were also reacted with outer membrane and secreted proteins from wild-type EHEC. Results were similar to those found for recombinant proteins, except that 2 control samples that did not react with recombinant intimin reacted with wild-type intimin.

Antibody response to *E. coli* O157 lipopolysaccharide in Canadian patients

Sera from the Canadian patients infected with *E. coli* O157:H7 were tested for the presence of antibodies to the lipopoysaccharide of *E. coli* O157 and compared with controls. In patient samples taken during the acute phase and after recovery, all but 1 acute sample and 2 convalescence samples were positive. All control samples were negative ($P<0.0001$). The assay of acute samples had a sensitivity of 90%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 91%.

Antibodies to Stx in Canadian patients

Sera from the Canadian children infected with *E. coli* O157:H7 were tested for antibodies to Stx and compared with controls. Immunoblot reactivity against Stx1 was found in 4 sera taken during the acute phase of the disease and 5 sera taken after recovery. Reactivity against Stx2 was found in 6 sera taken during the acute phase of the disease and 7 sera taken after recovery. Sera from pediatric controls exhibited reactivity to Stx1 (n=4) and Stx2 (n=12). Comparison of the antibody response in patients (considered positive if reactivity was detected during the acute or convalescent phase) with controls did not reach statistical significance. The assay of acute samples had a sensitivity of 40% for anti-Stx1 and 60% for anti-Stx2 and a specificity of 80% and 40% for anti-Stx1 and anti-Stx2, respectively. The positive predictive value was 50% and 33% and the negative predictive value 73% and 67%, respectively.

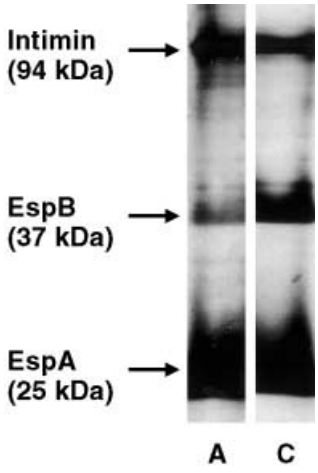

Fig. 2 Immunoblot of serum from a Canadian child with hemolytic uremic syndrome associated with *E. coli* O157:H7 run on a gel with recombinant intimin (94 kDa), EspA (25 kDa), and EspB (37 kDa). *A* Serum taken during the acute phase, *C* serum taken during convalescence

Antibody responses to EHEC antigens in Swedish patients

Sera from Swedish patients with HUS and HC exhibited an IgA, IgG, and IgM antibody response to EspA and EspB during the acute and convalescent phase of disease. In comparison to controls, significantly more patients developed antibodies to EspB. The antibody response to these proteins in patients, household members, and controls and statistical comparisons are summarized in Table 5. Antibodies to the EHEC antigens were found in patients with O157 and non-O157 infection as shown in Table 6.

Table 5 Antibodies to EHEC antigens in Swedish patients, household members, and controls

Patient or control group	Total number	EspA			EspB		
		IgA	IgG	IgM	IgA	IgG	IgM
HUS acute	30	24**	25**	27	19*	21*	19*
HUS convalescent	17	8	14**	11	4**	10**	8***
HC acute	8	4	7***	6	1	6**	4***
HC convalescent	2	1	2	2	1	2	1
Adult asymptomatic household members	21	16***	15	13	12**	14***	10
Pediatric asymptomatic household members	4	4***	4***	4	3*	4**	3***
Pediatric controls	35	12	12	30	0	5	5
Adult controls	17	6	7	7	0	5	3

*P<0.0001, **P<0.01, ***P<0.05 compared with controls. HUS patients were compared with pediatric controls, HC patients were compared with pediatric and adult controls combined, adult household members were compared with adult controls, and pediatric household members with pediatric controls

Table 6 Antibodies to EHEC antigens in HUS and HC patients with O157 and non-O157 infection upon admission

Serotype	Total number of patients[a]	EspA			EspB		
		IgA	IgG	IgM	IgA	IgG	IgM
O157	21	16	18	17	10	15	11
O121	4	4	4	4	2	4	3
O145	3	3	2	3	2	2	2
O103	1	0	1	1	0	1	1
O8	1	0	1	1	0	0	1
O26	1	0	0	1	1	0	1
O non-typeable, non-O157[b]	5	4	5	5	4	4	3

[a] Only patients from whom feces or serum were available during the acute phase of disease (for identification of the bacterial serotype by PCR and serotyping or by serodiagnosis of antibodies to EHEC LPS) are shown. Patients 5 and 11 (as in Table 3) are not shown, since the serotype of the infecting strain was not identified. Patients 2, 28, 33, and 34 are not shown since serum from the acute phase was not available

[b] In these patients the isolated fecal strain was non-typeable or analysis of the acute serum sample was negative for antibodies to O157-LPS (patients 7, 8, 18, 25, and 27 as in Table 3)

Comparison of the diagnostic value of the different antibody assays

The sensitivity, specificity, positive and negative predictive value of the assays for the detection of antibodies to EspA and EspB are presented in Table 7, in which results from all Swedish pediatric patients during the acute phase of disease and all pediatric controls are assessed. Comparison of the sensitivity and specificity of the various assays shows that EspA IgM gave the highest sensitivity (89%) and EspB IgA the highest specificity and positive predictive value (100% for both).

Correlation of antibodies to EspB with the severity of disease in symptomatic patients

Of the three EHEC antigens tested, antibodies to EspB were found to be the most specific for the diagnosis of recent EHEC infection. We therefore attempted to correlate the presence or absence of EspB antibodies during the acute phase of the disease with disease severity, as manifested by bloody diarrhea or renal failure in the Swedish patients with HUS and HC. Five patients lacked antibodies (IgA, IgG, and IgM) to EspB altogether; all 5 had bloody diarrhea and 4 required dialysis. Twenty-one patients lacked IgA antibodies to EspB (the 5 patients lacking IgA, IgG, and IgM are included here), 20 of these had bloody diarrhea, but only 11 required dialysis. Of the patients lacking IgA, there were 7 patients with HC (of a total of 8). Six patients with HUS did not develop renal failure and therefore did not require dialysis. Of these, 3 patients lacked IgA antibodies to EspB. In comparison, 11 of 21 patients that lacked IgA antibodies to EspB required dialysis and 16 of 17 that had IgA antibodies to EspB required dialysis (P<0.01), indicating that lack of IgA antibodies to EspB correlated with a milder form of disease. There was no correlation between the presence or absence of EspB IgM or IgG antibodies and the severity of intestinal or renal disease.

The presence of EspB IgG antibodies after recovery

We investigated the use of EspB IgG antibodies for the diagnosis of EHEC infection after recovery. Six of 10 Canadian convalescent sera (P<0.01 compared with controls, Table 4) and 10 of 17 Swedish convalescent sera (P<0.01 compared with controls, Table 5) contained IgG antibodies to EspB. Sera from 1 Canadian patient

Table 7 Diagnostic value of antibody assay for EHEC antigens

Diagnostic value for acute HUS and HC (%)[a]	EspA			EspB		
	IgA	IgG	IgM	IgA	IgG	IgM
Sensitivity	77	83	89	57	69	63
Specificity	66	66	14	100	86	86
Positive predictive value	69	71	51	100	83	81
Negative predictive value	74	79	55	70	73	70

[a] Sera from Swedish pediatric patients with HUS and HC (n=35) during the acute phase of disease and from Swedish pediatric controls (n=35) were compared. Only children were compared in order to achieve a better age match

taken 20 months after the acute phase still contained EspB IgG antibodies. Two Swedish patients seroconverted from EspB IgG negative to positive after recovery. Of the 2 Swedish patients whose sera were tested 10–11 years after acute HUS, 1 was EspB IgG positive and the other was negative.

Cost and time assessment

We assessed the cost of analyzing serum antibodies to EspB in an individual patient. Costs included running the purified His-tagged EspB on a ready-made SDS-PAGE gel, transfer, immunoblotting with patient serum, and detection by chemiluminescence (ECL kit, Amersham), but did not include technician time. The cost for each antibody test (IgA, IgG, or IgM) was equivalent to U.S. $2. The assay takes 1 working day to perform.

Discussion

EHEC antigens intimin, EspA, and EspB are required for bacterial attachment to the intestinal mucosa. In this study we have shown that patients with recent *E. coli* O157 and non-O157 infection develop an antibody response to these antigens. Based on statistical comparisons, specificity, and positive predictive value, EspB appears to be the most-appropriate assay for the detection of recent EHEC infection. We have compared this antibody response with that against *E. coli* O157 lipopolysaccharide, which is considered the standard serodiagnostic assay for infection with this serotype. Although the latter shows the highest sensitivity, specificity, and predictive values for the detection of EHEC O157 strains, there is no cross-reactivity with lipopolysaccharide antigens from other serotypes [5]. We have developed an assay that is not dependent on the serotype of the infecting strain and may thus be useful for the diagnosis of LEE-positive non-O157 infections.

Although *E. coli* O157:H7 appears to be the main serotype associated with HUS in the United States, Canada, and Western Europe [47, 48], many other serotypes have been identified in isolated cases and epidemics [37, 49, 50]. Serological diagnosis is useful in cases in which a fecal strain is not available, due to antibiotic treatment or a prolonged time lapse from the onset of symptoms, and in epidemics. The antibody response studied here utilized recombinant EHEC antigens that can be purified in considerably larger quantities than wild-type antigens. We have shown that sera from patients with EHEC O157 and at least five other serotypes react with these antigens. EHEC infection may thus be detected regardless of the serotype, as long as the strain produces intimin, EspA, and EspB. There is considerable sequence homology between the *eae* [19, 51, 52], *espA*, and *espB* [13, 19] genes of various EHEC and EPEC strains. Comparing EPEC O127:H6 with EHEC O157:H7, Perna et al. [33] found the LEE sequences to be 84.6% identical. Intimin from EPEC and EHEC strains has been found to cross-react with convalescent sera from a HUS patient infected with EHEC O111:H– [31] and serum from a HUS patient infected with EHEC O157:H7 reacted with EspA and EspB from EHEC O26:H11 and EPEC O127:H6 [15]. Furthermore, we have, both in this study and previously, presented evidence that wild-type EHEC proteins react with rabbit antiserum against the EPEC secreted proteins [15], and that polyclonal antibodies against EPEC antigens recognize the recombinant proteins. Taken together, these results imply that there is sufficient antigenic cross-reactivity between intimin, EspA, and EspB in various EHEC and EPEC serotypes and that the EHEC O157:H7 recombinant proteins developed in this study should be recognized by antisera from patients infected with EHEC strains of other serotypes. Due to the gene sequence homology between EPEC and EHEC strains, the presence of serum antibodies to EspB in isolated cases of diarrhea could indicate infection with either EHEC or EPEC strains. However, since EPEC do not cause HC or HUS, the presence of EspB antibodies in these conditions would indicate EHEC infection.

The major differences between the present study and those carried out earlier [15, 29, 31, 53, 54] are that larger numbers of patients were studied and they were infected with several non-O157 serotypes. The assay was shown to effectively detect IgA, IgG, and IgM antibodies to EspA and EspB in these patients. Furthermore, the recombinant proteins were prepared using an EHEC template, which may be of importance. Using recombinant EspB prepared from an EPEC template, Jenkins et al. [54] found that 2 of 7 patients with fecal carriage of

E. coli O157 had antibodies to EspB compared with 3 of 20 controls. They concluded that this might be due to lower levels of sequence homology between EPEC and EHEC EspB. In the present study we used recombinant EspB prepared from EHEC and found that 28 of 40 Canadian and Swedish pediatric patients developed an acute IgG antibody response to EspB compared with 7 of 55 Canadian and Swedish pediatric controls. Thus, using an EHEC template, the assay was found to have higher sensitivity and specificity.

A previous study examined the serum antibody response to Tir, EspA, EspB, and intimin in five HUS patients infected with *E. coli* O157:H7 and found increasing titers of all antigens, especially Tir, by day 8 of hospitalization [29]. Antigenic determinants in Tir are more heterogeneous than those in intimin, EspA and EspB. Immunoblot analysis of convalescent serum from HUS patients with EHEC strains O111:H– and O157:H– showed varying responses to Tir from strains O26:H–, O157:H–, and O111:H– [53]. In addition, there was a 56–97% sequence homology between the Tir sequences in various EHEC and EPEC strains [53]. Intimin, EspA, and EspB have a higher sequence homology between strains and are therefore more suitable for the study of serum responses in patients infected with different bacterial serotypes.

In epidemics of HC and HUS, in which a fecal strain has not been isolated, convalescent sera may be useful for the detection of previous EHEC infection. In this study convalescent sera from both Canadian and Swedish patients contained IgG antibodies to EspA and EspB. Convalescent sera from patients with HUS caused by LEE-positive EHEC strains contain antibodies to wild-type and recombinant Tir [53]. The recombinant Tir was highly susceptible to proteolytic degradation. In the present study we show that Intimin, EspA, and EspB were not subject to such breakdown, which may present a diagnostic advantage.

EHEC have been shown to produce an attaching and effacing lesion when binding to intestinal cells in in vitro organ cultures [55] and in animal models [18, 56, 57, 58], but this lesion has not yet been demonstrated in human disease. The results of this and previous investigations [15, 29, 31, 54] indicate that these antigens are expressed during natural human infection. The higher frequency of EspB IgM antibodies in patients than controls, and the seroconversion noticed in 2 patients when comparing acute and convalescent serum samples, indicate that the antibody response was related to an ongoing infection or one that occurred in the recent past.

A very low inoculum is sufficient in order to acquire EHEC infection [3, 59]. Person-to-person infection has been described and household members of a patient with symptomatic EHEC infection may thus become infected [60]. The majority of asymptomatic household members mounted an antibody response to the EHEC antigens EspA and EspB, suggesting that antigen expression does not necessarily lead to clinical symptoms.

The presence of IgA antibodies to EspB in symptomatic patients correlated with the severity of renal disease. We assume that IgA is generated in response to release of EspB in the gut, the larger the antigenic dose the greater likelihood of eliciting an immune response. Thus the intestinal inflammatory response may trigger the systemic host response, which, together with bacterial virulence factors, may contribute to more severe renal damage. In asymptomatic household members other defense mechanisms are presumably involved, preventing the development of symptoms in spite of infection. Consequently, the presence of IgA antibodies to EspB in asymptomatic individuals does not predict the development of disease, but may possibly reflect a booster antibody response to a past LEE positive infection. In the future we intend to develop an ELISA that will enable quantitative measurement of the antibody response to EspB. Differences between patients and family members regarding the amount of antibody may thus be found.

A relatively high number of controls had antibodies to EspA, intimin, and Stx. This may be due to prior contact with these antigens by infection with EPEC or EHEC strains. Children infected with EPEC strains have been shown to develop an antibody response to EspA and intimin [61]. Infection with EPEC or EHEC strains may account for antibodies to EspA and intimin in Canadian controls and antibodies to EspA in Swedish controls. Cross-reactivity with other undefined antigens may also contribute to the formation of these antibodies. The antibody response to EspB appears for this reason to be of higher diagnostic value. We suggest measurement of IgA and IgM antibodies to EspB as a diagnostic method with very high specificity that will enable detection of recent EHEC infection.

The data presented in this study indicate that antibody responses to EHEC antigens and especially EspB are elicited during EHEC infection and may be used for diagnostic purposes, specifically in cases in which a fecal strain has not been isolated or in epidemics.

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