Monoclonal antibodies against all known variants of EspA: development of a simple diagnostic test for enteropathogenic *Escherichia coli* based on a key virulence factor

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Received 25 March 2014
Accepted 16 September 2014

Enteropathogenic *Escherichia coli* (EPEC) are a major cause of infant diarrhoea in developing countries and a significant public health issue in industrialized countries. Currently there are no simple tests available for the diagnosis of EPEC. Serology of O-antigens is widely used routinely in many laboratories throughout the world, even though it has been known for many years to be an unreliable indicator of EPEC virulence. We have developed a simple, low-cost immunodiagnostic test based on the EspA filament, an essential virulence factor of EPEC and the related enterohaemorrhagic *E. coli* (EHEC). Using recombinant proteins of the five major variants of EspA as immunogens, we raised a panel of three monoclonal antibodies in mice that detects all variants of the native target in bacterial cultures. The antibodies proved suitable for application in sandwich-type assays, including ELISA and lateral flow immunoassays (LFI). Prototypes for both assays were specific for EPEC and EHEC strains when tested against a panel of control microorganisms. We have also developed a simple, affordable culture medium, A/E medium, which optimizes expression of EspA allowing improved sensitivity of detection compared with standard Dulbecco’s modified Eagle’s medium. Together these reagents form the basis of robust, informative tests for EPEC for use especially in developing countries but also for routine screening in any clinical laboratory.

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Abbreviations: A/E, attaching and effacing; CBT, Centre for Biotechnology; CMC, Christian Medical College; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; LFI, lateral flow immunoassay; NICED, National Institute of Cholera and Enteric Diseases; RKI, Robert Koch Institut; TMB, tetramethyl benzidine.

The GenBank/EMBL/DDBJ accession numbers for the 22 sequences of *espA* determined in this study are given in Table 2.
**INTRODUCTION**

Enteropathogenic *Escherichia coli* (EPEC) are a major cause of infant diarrhoea in developing countries, accounting for an estimated 10% of the approximately 1.4 billion paediatric diarrhoeal episodes annually in children under the age of 5 (O’Ryan et al., 2005). In the absence of treatment, particularly among very young children also affected by malnutrition, EPEC diarrhoea can be fatal or lead to irreversible damage to the intestine (Chen & Frankel, 2005). Industrialized countries have experienced an overall decline in childhood diarrhoea during the past 50 years, but EPEC still accounts for a similar proportion of diarrhoeal incidences (Afset et al., 2003; Robins-Browne et al., 2004). In addition in industrialized countries, the closely related pathotype enterohaemorrhagic *E. coli* (EHEC) is responsible for occasional, mainly food-borne outbreaks of diarrhoea in adults and children, frequently accompanied by severe complications such as haemorrhagic colitis and haemolytic uraemic syndrome due to the action of shigatoxins not present in EPEC (Frankel et al., 1998; Smith et al., 2004).

EPEC and EHEC are a heterogeneous group of *E. coli* strains. For many years the diagnosis of EPEC has been based primarily on the identification of O:H serotypes according to WHO guidelines dating from 1987, which recognized the 12 so-called classical EPEC serogroups associated with childhood diarrhoea: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Campos et al., 2004). EHEC strains are commonly associated with serogroups O103, O145 and O157, while some serogroups, in particular O55, O26 and O111, include both EPEC and EHEC strains. However, the serotyping scheme was developed before EPEC and EHEC virulence mechanisms were elucidated and many subsequent studies have shown that there is only partial correlation between serology and pathotype (Afset et al., 2003; Ajampur et al., 2008; Campos et al., 2004; Yang et al., 2007). Nevertheless, despite the fact that O-serology is non-informative of virulence, it continues to be used in many clinical laboratories throughout the world as one of the routine tests to establish the cause of diarrhoea (Kozub-Witkowski et al., 2008). While EHEC can be identified by immunological tests for shiga toxins, there is a real need for simple diagnostic tests for EPEC, based on known virulence factors, especially in developing countries where EPEC diarrhoea is endemic, but also in industrialized countries where studies indicate that EPEC may be more prevalent than was previously thought.

EPEC and EHEC colonize the intestinal epithelium causing attaching and effacing (A/E) lesions by a mechanism that involves the intimate attachment of bacteria to the host cell (Kenny et al., 1997). Various virulence factors essential for this process are encoded on a pathogenicity island, the locus of enteroocyte effacement, including intimin, a bacterial membrane adhesion protein encoded by the *eae* gene and the EspA (*E. coli* secreted protein A) filament, a hollow tube that acts like a molecular syringe for delivery of the Tir (translocated intimin receptor) protein and other effector molecules into the host cell (Crepin et al., 2005; Frankel et al., 1998; Knutton et al., 1998). Methods that target the presence of virulence genes, such as PCR and DNA microarray tests for the *eae* gene, are ideal as the basis for reliable diagnostic tests, but such methods are generally not applicable to routine diagnostic testing in peripheral health centres in developing countries where resources and skills may be limited. In these circumstances, simple antibody-based tests are much more suitable.

There have been several reports of antibodies raised against various secreted or surface-located EPEC virulence factors (Batchelor et al., 1999; Girón et al., 1995; Kühne et al., 2004; Lu et al., 2002; Menezes et al., 2010); however, these either detected only a limited subset of EPEC strains or required denaturation of the target for detection and to our knowledge have not been developed further. We chose the EspA filament, a 5-stranded helical polymer of identical 21 kDa monomers (Daniell et al., 2003; Delahay et al., 1999), as the immunological target for the development of a low cost EPEC diagnostic test initially intended for use primarily in the Indian subcontinent. We characterized *espA* gene sequences from a set of clinical isolates collected in south India and identified five major variants, all of which were represented, sometimes with minor variations, in the DNA and protein databases. Using recombinant proteins of these five variants as immunogens, we raised monoclonal antibodies capable of detecting all the EspA variants published to date. We also designed a low cost medium for optimal expression of EspA in culture. Together these reagents comprise a simple and reliable replacement for O-serogrouping for the identification of EPEC diarrhoea.

**METHODS**

**Bacterial strains and growth conditions.** Clinical isolates were obtained from the following laboratories: 16 *eae*+ strains from Christian Medical College (CMC), Vellore, India and four *eae*+ strains from the Centre for Biotechnology (CBT), Anna University, Chennai, India of known O:H serotype; 61 strains from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India, isolated on the basis of a positive PCR for intimin (*eae*+) but of unknown serotype; 242 strains from the Institut für Medizinische Mikrobiologie, Universität Leipzig, Germany, isolated on the basis of O-serogroups typical for EPEC and EHEC, and of these only 104 were *eae*+; 34 *eae*+ strains from the Department of Public Health, Faculty of Medicine, National Autonomous University of Mexico with known O:H serotypes; 14 *eae*+ strains from the Robert Koch Institut (RKI), Wernigerode, Germany, which had been O:H-serotyped and also tested for virulence factors associated with EHEC to distinguish EPEC (8 strains) from EHEC (3 *stx1* and 3 *stx2* strains). Non-EPEC reference strains (as listed in Fig. 6) were also from RKI.

Strains were maintained on Luria agar. To induce production of virulence factors, strains were inoculated into Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 1% glucose and incubated overnight at 37 °C in 5 ml volumes without agitation.

**Improved medium for virulence induction.** To improve the expression of EspA in some strains, we developed an alternative medium, A/E medium, based on soy peptone and yeast extract, using some poorly expressing strains as indicators of improvement in a dot blot
Taking into account the reported importance of calcium and sodium bicarbonate (Abe et al., 2002), and considering the components of DMEM that might be important, such as vitamins, we arrived at the following formulation: 4.5 g Difco Select Soytone l\(^{-1}\) (BD); 6 g HEPES l\(^{-1}\) (free acid; Melford); 2 g yeast extract l\(^{-1}\) (Oxoid); 10 g lactose l\(^{-1}\) (Fisher Scientific); 0.2 g CaCl\(_2\) . H\(_2\)O l\(^{-1}\) (Sigma); 0.2 g ferric ammonium chloride l\(^{-1}\) (Sigma); and 0.4 g KCl l\(^{-1}\) (Sigma). The medium was sterilized either by autoclaving or by filter-sterilization. NaHCO\(_3\) powder (Sigma) was added to a final concentration of 7.5 g l\(^{-1}\) immediately prior to use. Cultures (5–10 ml) were inoculated and incubated overnight at 37°C without agitation.

**Cloning and mutagenesis.** Variant espA genes were amplified by PCR from genomic DNA using flanking primers UP1 F/UP1 R (727 bp) or UP2 F/UP2 R (1010 bp; Table 1). Products were purified from agarose gels and the DNA sequences determined using the same primers. For cloning and expression of recombinant proteins, espA coding regions were amplified from five strains using primers EspA F1 and EspA R1 (isolate III-3, EspA\(\alpha\); 591 bp); EspA F2 and EspA R2 (isolate A5, EspA\(\beta\) and isolate A7, EspA\(\delta\); 592 bp); EspA F3 and EspA R2 (isolate III5, EspA\(\gamma\); 592 bp) and EspA F6 and EspA R4 (isolate C2; EspA\(\varepsilon\); 582 bp). PCR products were digested with the appropriate restriction enzymes (as indicated in Table 1) and cloned into similarly cut vector pET28a (Novagen) in E. coli strain XL10 Gold (Stratagene). After confirmation by DNA sequencing using primer T7 (homologous to vector sequence) the recombinant plasmids were transformed into strain BL21(DE3) (Stratagene).

For epitope mapping, overlapping fragments of the espA genes from the three variants representing the three mAbs used in this work were cloned into pET28a. Templates for amplification were recombinant plasmids containing EspA\(\beta\) for mapping mAb 14, EspA\(\delta\) for mapping mAb 209 and EspA\(\varepsilon\) for mapping mAb 2. EspA\(\beta\) fragments were amplified using primer combinations Epi F21/Epi R12 (299 bp; amino acids 110–192) and Epi F20/Epi R1 (371 bp; amino acids 1–118). EspA\(\delta\) fragments were amplified with primers Epi F12/Epi R10 (428 bp; amino acids 1–137) and Epi F21/Epi R12 (299 bp; amino acids 100–192). EspA\(\varepsilon\) fragments were amplified with primers Epi F6/Epi R9 (422 bp; amino acids 1–135), Epi F7/Epi R5 (356 bp; amino acids 78–190) and Epi F6/Epi R20 (485 bp; amino acids 1–156). Fragments were digested with restriction enzymes as indicated in Table 1 and cloned into similarly digested pET28a.

EspA mutant proteins containing single amino acid changes were produced by first amplifying two separate PCR fragments primed
Table 1. Primers for cloning and mutagenesis

| Primer     | Sequence (5’ to 3’)                      | $T_m$ (°C) |
|------------|------------------------------------------|------------|
| UP1 F      | TAATACATTATTAATGATTGTTAAAG              | 54.8       |
| UP1 R      | TATCGYTATTTACRTTAAGTAGT                 | 56.6       |
| UP2 F      | CTCGGGTAGCTGTTGTCGAGGT                | 67.4       |
| UP2 R      | CAGAGGGGCGTCATCAATGATG                 | 66.2       |
| EspA F1 (x; Ndel) | GCCATATGGATACATCACTACACTACGAC    | 67.2       |
| EspA R1 (x; Saci) | GCGAGCTCTTTATTTACCAAGGGATATTCTCG   | 70.0       |
| EspA F2 (β; β; Ndel) | GCGATATGGATACATCAGTCACCACCTC   | 70.4       |
| EspA R2 (β; β; Saci) | GCGAGCTCTTTATTTACCAAGGGATATTCTCG | 72.7       |
| EspA F6 (ε; Not) | CAACATGGATATCAGATATCAGTCT   | 65.2       |
| EspA R4 (ε; Xhol) | CGCTCGAGTTTACAAAAACTTATTGC   | 70.4       |
| EspA F5 (ε; Ndel) | GCCATATGGATACATCAGTCACACGAC   | 70.1       |
| Epi F21 (β; BamHI) | GCGGATCCATGAAAAGCCTACCTCCTCA   | 77.6       |
| Epi R12 (β; β; HindIII) | GCAAAGCTTTATTTACCAAGGGATATTG   | 65.4       |
| Epi F20 (β; BamHI) | GCGGATCCATGATACATCAGTCACACG   | 72.2       |
| Epi R1 (β; HindIII) | GCAAGTTAAATTTACCAAGGGATATTG   | 73.5       |
| Epi F12 (δ; BamHI) | GCGGATCCATGATACATCAGTCACACG   | 72.2       |
| Epi R10 (δ; HindIII) | GCAAGCTTTATTTACCAAGGGATATTG   | 80.1       |
| Epi F6 (ε; BamHI) | GCGGATCCATGATACATCAGTCACACG   | 72.1       |
| Epi R9 (ε; HindIII) | GCAAGCTTTATTTACCAAGGGATATTG   | 78.7       |
| Epi F7 (ε; BamHI) | GCGGATCCATGATACATCAGTCACACG   | 83.3       |
| Epi R5 (ε; HindIII) | GCAAGCTTTATTTACCAAGGGATATTG   | 64.7       |
| Epi R20 (ε; HindIII) | GCAAGCTTTATTTACCAAGGGATATTG   | 70.3       |
| Mut 1 (β N112K) | GTGATTCGACTTATAAGATCGTCGTCAATG    | 67.7       |
| Mut 2 (β N112K) | CATTGCGAGGTATTTAAATATGCAC        | 67.7       |
| Mut 15 (β D117G) | CTATATAATAGTCCCTGCAATGCGATTACA...   | 76.8       |
|             | ...GTAAGTGGTATTGC                   |            |
| Mut 16 (β D117G) | CGCTAAATACCACTACCTGTAATGCGATTGCC  | 76.8       |
|             | ...AGGATCTATTATATAG                 |            |
| Mut 5 (δ N96E) | GTTCAGGACGTTCAGGAAGATCGAAAGGCGAAACCT | 80.2       |
| Mut 6 (δ N96E) | AAGGTTGCAGCTTTACCCATTACGACGATCTCAAGT | 80.2       |
| Mut 7 (δ K112Q) | GTGATCGACTTATAAGATCGTCGTCAATG    | 72.2       |
| Mut 8 (δ K112Q) | GTAATAGCCATGATCGGTATGTAATAGCGATC  | 72.2       |
| Mut 17 (ε E94D) | CTGAACTGACCTGCTAGCCGAAAGGAATTAC... | 77.0       |
|             | ...AAGGCTACCTTC                   |            |
| Mut 18 (ε E94D) | GAAGGTTTCAGCTTTACCCATTACGCTCGAAT... | 77.0       |
|             | ...GACTGAACTCAG                   |            |
| T7         | AATACGACTCACTATAGGG               | 51.2       |

Where appropriate, primer names indicate EspA type and restriction sites (in italics) used for cloning into vector DNA. Primers used for mutagenesis (Mut) also indicate the amino acid substitution achieved (underlined). $T_m$ values are based on the $T_m$ Calculator from Thermo Scientific.

with overlapping internal primers containing the appropriate base changes and external primers, and then mixing the resulting fragments together and amplifying with external primers only. The resulting products were digested with restriction enzymes, cloned and expressed in pET28a using the following combinations: EspA β (N112K) Mut 1/EsPa R2 (268 bp) and Mut 2/EsPa F2 (354 bp); EspA β (D117G) Mut 15/EsPa R2 (261 bp) and Mut 16/EsPa F2 (378 bp); EspA δ (N96E) Mut 5/EsPa R2 (317 bp) and Mut 6/EsPa F2 (313 bp); EspA δ (K112Q) Mut 7/EsPa R2 (269 bp) and Mut 8/EsPa F2 (361 bp); EspA ε (E94D) Mut 17/EsPa R4 (319 bp) and Mut 18/EsPa F6 (308 bp).

Recombinant protein expression and purification. Recombinant plasmids were transformed into BL21(D3) (Stratagene) by the calcium chloride method. Transformants were grown at 37 °C to mid-exponential phase in 400 ml Luria broth, induced by addition of IPTG to 1 mM and grown for a further 4 h. Recombinant proteins were isolated in the form of inclusion bodies. Cells in 50 ml culture volumes were pelleted by centrifugation and resuspended in 2.5 ml 0.1 M sodium phosphate (pH 8.5), 0.3 M sodium chloride. After incubation for 30 min at 37 °C in the presence of lysozyme (10 mg ml⁻¹) the cells were disrupted by sonication on ice. Suspensions were centrifuged at 6000 r.p.m. for 10 min in an Eppendorf Minispin centrifuge and pellets were resuspended in wash buffer (50 mM Tris, pH 8, 0.1 M sodium chloride, 2 M urea, 0.5% Nonidet) by syringing to break up clumps. Washing was repeated and suspensions were stored in wash buffer at −20 °C overnight, resulting in almost complete clearing of the suspension. Suspensions were centrifuged at 13500 r.p.m. for 5 min and the clear supernatants were removed and dialysed against several changes of PBS. Essentially pure EspA protein, which was
recovered as a fine translucent precipitate, was quantified by SDS-PAGE against dilutions of a BSA standard.

**mAb production.** The five major espA variants (as shown in Fig. 2) were used as targets for raising monoclonal antibodies. BALB/c mice were immunized with recombinant EspA proteins from different EPEC strains, presented in the form of inclusion bodies, either individually or as mixtures, in the presence of Freund’s adjuvant or Titermax and boosted on two or three subsequent occasions at two-weekly intervals. Serum titres were determined by whole cell ELISA and positive wells were cloned twice and expanded for processing as for Western blots.

**Direct whole cell ELISA.** EPEC strains expressing EspA variants were grown overnight in DMEM containing 1% glucose. Cultures were either mixed together in equal proportions (for initial screening of hybridomas) or used separately (for subsequent testing) and diluted with an equal volume of coating buffer. For testing, 100 μl of mixture was dispensed into each well of 96-well microtitre plates (NUNC Maxisorp), dried overnight at 37°C, washed twice with PBS and then blocked for 30 min with PBS containing 4% skimmed milk. Doubling dilutions of serum from test bleeds or undiluted hybridoma supernatants were added to wells and incubated for 1 h, washed three times with PBS, incubated with goat antimouse alkaline phosphatase (Sigma) for 30 min and then washed five times with PBS. Detection was by p-nitrophenyl phosphate (Sigma); reactions were stopped by the addition of 1/4 volume of 3 M sodium hydroxide and absorbance was read at 405 nm.

**Sandwich ELISA.** Purified mAbs were conjugated to horseradish peroxidase using the Lightning-Link kit (Innova Biosciences) according to the manufacturer’s instructions. Microtitre plates were coated with purified mAbs, either individually or as a mixture, at 10 μg ml⁻¹ in coating buffer and stored at 4°C overnight. After rinsing twice with PBS the plates were blocked with 4% skimmed milk in PBS for 30 min. Overnight cultures were added to wells either directly or after dilution with PBS and incubated for 1 h. After five washes detection was by horseradish peroxidase-conjugated mAbs, again either separately or as a mixture, followed by substrate TMB (liquid substrate system for ELISA, Sigma). After 15 min results were recorded by scanning.

**Western blots and dot blots.** Proteins were resolved on 12% polyacrylamide gels and blotted onto nitrocellulose membranes. These were blocked in PBS containing 4% skimmed milk and probed with hybridoma supernatants at dilutions of 1:100 to 1:500 for 1 h. Bound antibody was detected with goat antimouse horseradish peroxidase (Sigma) for 30 min and then washed five times with PBS. Detection was by p-nitrophenyl phosphate (Sigma); reactions were stopped by the addition of 1/4 volume of 3 M sodium hydroxide and absorbance was read at 405 nm.

**Fig. 2.** Alignment of five representative EspA amino acid sequences used for raising mAbs, using CLUSTAL 2. Source strains are A5, EspA α; A7, EspA δ; III3, EspA β; III5, EspA γ; C2, EspA ε. Shading indicates residues that differ from the consensus.

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R-Biopharm prototype sandwich ELISA. The sandwich ELISA was carried out in 96-well plates with 12 separate strips of eight wells each in a strip holder, which were supplied ready coated with a mixture of mAbs, stabilized and blocked. All solutions except wash buffer and sample diluent were provided in dropper bottles. To carry out the test, samples were diluted with an equal volume of diluent (protein-buffered NaCl solution) and 100 µl was applied to each well, followed immediately by two drops of conjugate 1 (biotin-conjugated mAbs in stabilized protein solution). After incubation for 1 h at room temperature, wells were rinsed five times with wash buffer (PBS containing 0.1% thimerosal) and incubated for 30 min with two drops of conjugate 2 (streptavidin-enhanced horseradish peroxidase conjugate in stabilized protein solution). After five washes two drops of substrate (hydrogen/TMB ready to use) were added and left to incubate for 15 min. Results were recorded either by scanning for a visual record, or by stopping the reaction with one drop of stop solution (2 M H2SO4) followed by measurement of absorbance at 450 nm.

Lateral flow immunoassay (LFI). A prototype LFI was developed in collaboration with Forsite Diagnostics Ltd, York, UK. Purified mAbs were adsorbed to blue latex beads and incorporated into the release pad. The capture antibody was line sprayed onto Prima 40 nitrocellulose membranes (GE Health Care), initially using a single antibody for each device. A control line to indicate a functional assay contained the EspA sequences of strains from south India, exemplified by the well-characterized serotypes O111: H2 and O127: H6 (streptavidin-enhanced horseradish peroxidase conjugate in stabilized protein solution). After five washes two drops of substrate (hydrogen/TMB ready to use) were added and left to incubate for 15 min. Results were recorded either by scanning for a visual record, or by stopping the reaction with one drop of stop solution (2 M H2SO4) followed by measurement of absorbance at 450 nm.

RESULTS

EspA sequences of strains from south India

The EspA proteins encoded by the 21 confirmed EPEC or EHEC strains from south India (Table 2) were found to be either identical or closely related to published EspA protein sequences. A phylogenetic tree illustrating the relationship of these EspA proteins to those in protein and DNA databases is shown in Fig. 1, which also includes the EspA sequences of B155, an A/E lesion-forming strain of unknown serotype from NICED (see below) and Citrobacter freundii, an organism that is commonly found in soil and water but may be present in the intestinal tract of humans and animals (Tschäpe et al., 1995).

To rationalize the nomenclature of EspA we have assigned the sequences to five groups based on similarity and designated them using letters of the Greek alphabet by analogy with the nomenclature for intimin. Three of these groups, EspA α, EspA β and EspA γ, exemplified by the well-characterized serotypes O127: H6, O111: H2 and O157: H7, respectively, had already been designated as such in accordance with the intimin variants carried by these strains. We suggest the designation EspA δ for the group of variants including O49: H12, which was previously named EspA ‘beta variant’ (Bertin et al., 2004), and EspA ε for the group including O119: H6 that showed the greatest sequence divergence of all variants and which had been variously called EspA ‘beta variant’ or EspA ‘β2’ (Afset et al., 2006; Garrido et al., 2006).

Table 2. Clinical isolates from India

| Leic. no. | Collection | Serotype | EspA type | GenBank |
|----------|------------|----------|-----------|---------|
| III 3*   | RKI 05-02944 | O127: H6 | α         | KJ549668 |
| B5       | RKI 04-02142 | O127: H6 | α         | KJ549669 |
| E4       | RKI 04-02177 | O127: H6 | α         | KJ549670 |
| F6       | RKI 04-02191 | O127: H6 | α         | KJ549671 |
| G6*      | RKI 04-02203 | O55: H2  | β         | KJ549672 |
| B3       | RKI 04-02140 | O55: H2  | β         | KJ549673 |
| H1*      | RKI 04-02210 | O128: H2 | β         | KJ549674 |
| G1       | RKI 04-02198 | O128: H2 | β         | KJ549675 |
| A5*      | RKI 04-02130 | O126: H2 | β         | KJ549676 |
| H3       | RKI 04-02212 | O126: H2 | β         | KJ549677 |
| III 5*   | RKI 05-2946 | O157: H7 | γ         | KJ549678 |
| III 14   | RKI 05-2948 | O157: H7 | γ         | KJ549679 |
| III 6*   | RKI 05-2947 | O55: H2  | γ         | KJ549680 |
| H2*      | RKI 04-02211 | O125: H2 | δ         | KJ549681 |
| A6       | RKI 04-02131 | O125ab: H2 | δ     | KJ549682 |
| A7*      | RKI 04-02132 | O26: H2  | δ         | KJ549683 |
| C2*      | RKI 04-02151 | O119: H6 | ε         | KJ549684 |
| G2       | RKI 04-02199 | O119: H6 | ε         | KJ549685 |
| D2*      | RKI 04-02163 | O86: H34 | ε         | KJ549686 |
| F5       | RKI 04-02190 | O55: H2  | ε         | KJ549687 |
| H6       | RKI 04-02215 | O55: H2  | ε         | KJ549688 |
| B155*    | NICED B155  | ND       | ε         | KJ549689 |

Strains were collected at CBT and CMC; B155 was from NICED. All except B155 were logged and serotyped at RKI and espA DNA sequences were determined at the University of Leicester. Deduced EspA protein sequences were classified into five subtypes according to similarity and denoted by α, β, γ, δ or ε.

*Strains included in the phylogenetic tree shown in Fig. 1: O55 non-typosable for O-antigens of classical EPEC/EHEC strains (O26, O55, O86, O103, O111, O114, O119, O125, O127, O128, O126, O142 O145, O157); ND, not determined.

Production and characterization of mAbs

We chose representatives of the five major EspA proteins (Fig. 2) as targets for the production of mAbs. The initial screen of hybridoma supernatants by whole cell ELISA was designed to select only those reacting with the native target. The resulting antibodies gave various patterns of cross-reactions with the different EspA types. Three hybridomas which produced strong signals in various applications and together detected all EspA variants were selected for further work.

Western blots against recombinant EspA confirmed that the positive ELISA signals were due to specific interactions with EspA protein (Fig. 3a, b); mAb 14 reacted strongly with EspA types α, β and γ and weakly with EspA δ, while mAb 2 and mAb 209 detected one EspA type each, ε and δ.
respectively. Recombinant EspA ε produced multiple bands visible in both the Coomassie stained gel and a Western blot with mAb 2; unlike the other recombinant EspA variants, EspA ε contains a C-terminal histidine tag which may have resulted in translation initiation at downstream methionine codons within the espA gene. Western blots of cell lysates from corresponding overnight cultures of EPEC strains confirmed detection of a single band migrating at the expected position for a 21 kDa protein (Fig. 3c). The pattern of reactivity was similar to that with recombinant proteins, although no cross-reaction of mAb 14 was seen with a lysate of strain H2 carrying EspA d.

Similar patterns of reactions were obtained with two versions of a direct whole cell ELISA of immobilized cultures, using either horseradish peroxidase-conjugated secondary antibody for rapid visual inspection of results, or alkaline phosphatase-conjugated secondary antibody for absorbance measurement using a plate reader (Fig. 3d, e). Strain B155, an A/E lesion-forming strain of unknown serotype from NICED, was included here as it was unexpectedly detected by both mAb 209 and mAb 209.

For testing large numbers of samples simultaneously during the development phase of the work, we used a dot blot. This gave results essentially identical to those obtained with ELISA (Fig. 3f), although mAb 209 also showed a slight cross-reaction in this test with strain C2 carrying EspA ε.

**Improved medium for EspA induction**

DMEM has traditionally been used to study the virulence factors of EPEC, but we found it to be suboptimal for EspA expression in some strains in our collections. DMEM is also likely to be too expensive for routine use in developing countries. We therefore developed an alternative cheaper medium, which we called A/E (for attaching and effacing) medium, that enhanced the detection of EspA after overnight growth compared with DMEM for a representative sample of strains (Fig. 3g). In a time-course of EspA induction, most of these strains were detectable by dot blot after 4 h and all strains after 5 h in A/E medium, starting with a 1/100 dilution from an overnight culture in Luria broth (data not shown).

**Epitope mapping and IgG isotyping**

Overlapping fragments of espA from isolates A5 (β), H2 (δ) and C2 (ε) were tested by dot blots with mAbs 14, 209 and 2, respectively (Fig. 4a). Corresponding Western blots (not shown) confirmed that the signal was specific for EspA...
In the case of mAb 14 the complete antigenic region was present on a short peptide between amino acids 100 and 118; the other two required larger fragments, including the central variable region of the EspA protein, for full recognition by the cognate antibody.

Individual amino acid residues were chosen for mutagenesis of the three EspA targets on the basis of sequence homology in the central region and antibody cross-reactions. The full-length mutated EspA proteins were tested in dot blots (Fig. 4a) and Western blots (not shown) as above. In all three cases individual and mixed antibodies for capture and detection. The most specific antibody was mAb 2 (ε) also significantly cross-reacted with EspA ε. The specific antibody was mAb 2 (ε). The prototype ELISA developed by R-Biopharm AG uses a mixture of all three antibodies for both capture and detection.

In initial tests with the LFI devices we occasionally observed false positive bands at the test line. This is illustrated in Fig. 5b, where strain III20, an Orough : H6 strain from the CBT collection (RKI no. 05-02949) that carries an espA deletion, gave no signal by dot blot but a positive test line by LFI, however removal of bacterial cells by brief centrifugation yielded a supernatant that was negative in both tests. By contrast, strains A5, C2 and H2, which express EspA proteins, gave positive signals in both tests with or without centrifugation. Subsequent testing of many other EPEC and EHEC strains confirmed that most, if not all, of the antigenic activity was present in the culture supernatant after overnight growth, suggesting that prior centrifugation could be used to prevent false positive results.
Specificity and sensitivity

To determine the specificity of our mAbs we tested a panel of reference strains held in the collection at RKI by dot blot and sandwich ELISA following growth in A/E medium (Fig. 6a, b). While all known EPEC (n=4) and EHEC (n=3) strains gave positive results in both tests, all strains of the following species tested were negative: *Aeromonas hydrophila*, *Bacillus cereus*, *C. freundii*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, *Morganella morganii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Salmonella Enteritidis*, *Salmonella Typhimurium* and *Shigella boydii*.

To determine the relative sensitivity of the various platforms, cultures were grown overnight in A/E medium and serial dilutions in PBS were tested by dot blots and by the prototype ELISA and LFI kits (Fig. 7a–c). ELISA showed much greater sensitivity than the other two tests, with dilution of cultures to at least 160-fold giving positive signals with all strains. Strains C2 and B155, recognized by mAb 2, showed even greater detectability to a dilution of 640-fold.

Coverage of mAbs

We tested all *eae*+ strains in our collection for EspA detection after overnight growth in A/E medium using the three antibodies separately in triplicate dot blots. The results are summarized in Table 3. Among the 213 *eae*+ isolates, 210 were detected with at least one antibody. Seven strains from NICED (including B155) were detected by both mAb 2 and mAb 209; DNA sequencing confirmed that their espA genes were identical to that of B155, encoding the variant of EspA containing both essential amino acids for recognition by the two mAbs. Of the three dot blot negative
strains, one was a known EHEC strain from RKI, serotype O26:H–, one was a strain from Leipzig assigned to pool 1 O-antigens associated predominantly with EHEC and the third was a strain from NICED of unknown serogroup. Amplification of espA DNA and sequencing of products confirmed the presence of intact coding regions for all three strains: EspA γ for the Leipzig and NICED strains and a β type for the RKI strain. Subsequent testing of these strains by the more sensitive R-Biopharm ELISA also gave negative results suggesting that EspA protein was not expressed sufficiently in these strains to be detected under these conditions.

Lack of correlation between serology and A/E virulence

The heterogeneity of EspA types within EPEC and EHEC serogroups is illustrated in Table 4. Of the 242 strains from Leipzig, isolated on the basis of serotypes classically associated with EPEC and EHEC, only 104 were shown to be eae+ (intimin-positive) by PCR. The majority of these belonged to O-serogroups such as O26, O55 and O157 that are traditionally, although not exclusively, associated with EHEC rather than EPEC. Similarly, in a recent trial of the R-Biopharm ELISA kit with clinical isolates from children with diarrhoea at CMC, Vellore, only 3 out of 20 strains characterized as EPEC by serotyping were actually positive for EspA and subsequent testing of the samples by PCR confirmed the presence of eae in these three strains, but not in any of the other strains (Table 4). These data confirm the much-reported poor correlation between E. coli O-serogroup and virulence.

DISCUSSION

The EspA filament is an excellent immunodiagnostic target for infant diarrhoea caused by EPEC. It is a key virulence factor present in all EPEC strains, it can be induced to high levels in culture and its structure makes it accessible from all sides to antibodies. We have raised a panel of three monoclonal antibodies that together recognize all five currently known major variants of EspA (α, β, γ, δ and ε) found in EPEC and EHEC strains. Perhaps not surprisingly, one mAb detected the three most closely related EspA types α, β and γ, whereas specific antibodies were elicited by the more divergent EspA variants δ and ε. The three antibodies cross-reacted in Western blots with purified recombinant EspA proteins and with extracts from cultures grown in medium that induced the expression of EspA. The pattern of cross-reactions of antibodies with EspA variants was identical in ELISA using immobilized cultures. Importantly, while almost all strains known to be EPEC, on the basis of PCR of the intimin gene eae, expressed a protein that was recognized by one or more of our antibodies, none of a panel of non-EPEC/non-EHEC commensal and pathogenic intestinal reference strains were detected by the EspA antibodies in any test format.

EspA proved a suitable target for sandwich-type tests using the same antibody for capture and detection. In such a test only multimeric targets can be detected with a single mAb and although EspA filaments were found to be detached from the EPEC cells after overnight growth it is clear that they must remain at least partially intact to be detected in both the sandwich ELISA and LFI. The sandwich ELISA was by far the most sensitive test, even when mixtures of antibodies for capture and detection were used. Of the three mAbs used, mAb 2 gave the strongest signal for its target in all test formats, so there may be a need to adjust the proportions of the antibodies in mixed assays to optimize sensitivity. However, even before optimization,
dilution of EPEC cultures up to 1/160 could be reliably detected by the sandwich ELISA. Given that an overnight culture of EPEC typically reaches a density of approximately $10^9$ cells ml$^{-1}$, the limit of detection for the most sensitive test format is therefore $10^6$ to $10^7$ cells ml$^{-1}$.

Results with LFIs suggest that optimization of this platform would present more of a challenge as some non-EPEC control cultures gave false positive test lines, presumably due to bacterial aggregates. This problem was solved by prior centrifugation and since the antigenic activity of EPEC cultures was present in the supernatant, a centrifugation step could safely be added to prevent false positive results without compromising the efficacy of the test. The disadvantage of introducing this additional step, however, is that it significantly reduces simplicity and convenience, which are important considerations for the adoption of the test, especially in developing countries. It may be possible to avoid this centrifugation step by using a support matrix other than nitrocellulose that does not impede the migration of bacterial cells and aggregates, but this has not yet been tested. However, the main disadvantage of LFI compared with sandwich ELISA remains its much lower sensitivity, giving positive test lines only up to ten-fold dilutions for some of the strains with the mixed antibody devices.

A crucial factor determining the sensitivity of the antibody-based tests was the growth medium used for inducing EspA expression. We noticed great variations in the expression of EspA using DMEM as the culture medium and we used some of the poor expressers as indicator strains to formulate an improved medium, A/E medium, which gave good results with many strains that had previously been undetectable or that showed very weak signals in the dot blot when grown in DMEM. Among 213 clinical isolates showing a positive PCR signal for the intimin gene $eae$, only three were not detected by our antibodies after growth in A/E medium, even with the most sensitive ELISA test. One of these was a confirmed EHEC strain and the other two belonged to O-serogroups or carried the $c$ variant of EspA often associated with EHEC. All three had intact $espA$ coding regions, so the lack of EspA protein must have been the result of $espA$ gene repression. Differences in virulence regulation between EPEC and EHEC have been reported (Mellies et al., 2007). Nevertheless, we were able to detect five other confirmed EHEC strains from the RKI collection, giving positive test lines only up to ten-fold dilutions for some of the strains with the mixed antibody devices.

### Table 3. Detection of intimin-positive isolates with EspA mAbs

| Serogroup | 14 ($\alpha,\beta,\gamma$) | 2 ($\alpha$) | 209 ($\delta$) | 2 + 209 ($\alpha, \delta$) | None | Total |
|-----------|-------------------------|-------------|---------------|-------------------------|------|-------|
| O26       | 18                      | 1           | --            | --                      | 1 ($\beta$) | 20    |
| O55       | 33                      | 1           | 2             | --                      | --   | 36    |
| O86       | --                      | 1           | --            | --                      | --   | 1     |
| O103      | 8                       | --          | 5             | --                      | --   | 13    |
| O111      | 11                      | --          | --            | --                      | --   | 11    |
| O113      | --                      | 1           | --            | --                      | --   | 1     |
| O114      | 3                       | 6           | --            | --                      | --   | 9     |
| O118      | 1                       | 2           | --            | --                      | --   | 3     |
| O119      | 2                       | 6           | --            | --                      | --   | 8     |
| O125      | 1                       | --          | --            | --                      | --   | 1     |
| O126      | 2                       | --          | --            | --                      | --   | 2     |
| O127      | 5                       | --          | 1             | --                      | --   | 6     |
| O128      | 9                       | 1           | --            | --                      | --   | 10    |
| O142      | 1                       | --          | --            | --                      | --   | 1     |
| O145      | 12                      | 3           | --            | --                      | --   | 15    |
| O157      | 7                       | --          | 5             | --                      | --   | 12    |
| Untyped   | 32                      | 15          | 6             | 7                       | 1 ($\gamma$) | 61    |
| Pool 1*   | --                      | --          | --            | --                      | --   | 1     |
| Pool 2†   | 1                       | --          | --            | --                      | --   | 1     |
| Pool 3‡   | 1                       | --          | --            | --                      | --   | 1     |
| Total     | 147                     | 37          | 19            | 7                       | 3    | 213   |

Intimin-positive strains from Leipzig, RKI, Mexico and NICED were grown in A/E medium overnight and aliquots were tested in triplicate dot blots, each probed with a separate mAb. For strains not detected by an mAb, the EspA type derived from DNA sequencing of the $espA$ genes is indicated in parentheses.

*O26, O103, O111, O145, O157.
†O55, O119, O125ac, O127, O128ab.
‡O86, O114, O126, O142.
as well as a large number of likely EHEC isolates in the Leipzig collection. Indeed, 75 of 104 intimin-positive strains from Leipzig belonged to serogroups usually associated with EHEC or shared between EPEC and EHEC.

Our screening results with clinical isolates provide further evidence for the lack of reliability of serological classification as a method of determining pathotypes of *E. coli* strains. It is clear that on the one hand a large proportion of samples are currently misdiagnosed as EPEC by serogrouping, while on the other hand an unknown number are not detected. This highlights the superiority of our immunological test for routine diagnostic purposes. Our panel of three antibodies detects all currently known variants of EspA, including minor local variants such as the unusual e variant from NICED that reacted with two of our antibodies.

In summary, when used together with the improved medium for EspA induction the prototype sandwich ELISA based on a panel of three monoclonal antibodies was shown to be specific and sensitive and thus has potential as a routine diagnostic tool for the identification of EPEC. Where EHEC rather than EPEC is suspected this can be confirmed by a parallel test for shigatoxins. The level of sensitivity is sufficient for testing mixed cultures grown from stool samples of children with diarrhoea using simple visual interpretation of results without the need for a plate reader. Further work is underway in our laboratories to develop a routine protocol for the detection of EPEC in stool samples. The prototype LFI was less sensitive than the ELISA platform and so is not as promising for the identification of EPEC in mixed cultures. However, for reference and diagnostic laboratories working with pure cultures our LFI represents a valuable, informative and reliable tool in the repertoire of diagnostic tests, particularly as a replacement for conventional serology.

**ACKNOWLEDGEMENTS**

This work was funded by EU grant ‘EACHChild’ ICA4-CT-2002-10032, and by the Wellcome Trust Translational award ‘Development of a simple rapid test kit for the early diagnosis of enteropathogenic *Escherichia coli* (EPEC) in children with diarrhoea’. We would like to thank Gad Frankel for the gift of polyclonal anti-EspA antiserum used in the exploratory stages of this work; Jennifer Bailey for excellent technical assistance; Chris Danks for expert advice on lateral flow technology; and Julie Pratt for critical reading of the manuscript. Conflict of interest: the materials described have been licensed to R-Biopharm, and in case of commercialization, any royalties due are split between U.P., R.R., P.W., the Wellcome Trust and the University of Leicester.

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**Table 4. Frequency of intimin-positive strains among O-serogroups classically associated with EPEC and EHEC**

| Serogroup | IMM, Leipzig | CMC, Vellore |
|-----------|--------------|--------------|
|           | Intimin-positive | Intimin-negative | Intimin-positive | Intimin-negative |
| O55       | 25            | 6             | 1              | 3             |
| O128      | 9             | 22            | –              | –             |
| O26       | 16            | 3             | 2              | 7             |
| O125      | 1             | 18            | –              | –             |
| O126      | 0             | 18            | 0              | 3             |
| O145      | 13            | 4             | –              | –             |
| O103      | 12            | 4             | –              | –             |
| O86       | 1             | 15            | –              | –             |
| O114      | 7             | 8             | –              | –             |
| O127      | 3             | 10            | 0              | 1             |
| O157      | 8             | 0             | –              | –             |
| O111      | 0             | 8             | 0              | 3             |
| O119      | 3             | 3             | –              | –             |
| O118      | 3             | 0             | –              | –             |
| O142      | 0             | 3             | –              | –             |
| O158      | 0             | 2             | –              | –             |
| Pools 1,2,3* | 3          | 14            | –              | –             |
| Total     | 104           | 138           | 3              | 17            |

Heterogeneity of EspA types within EPEC and EHEC serogroups in strains from IMM, Institut für Medizinische Mikrobiologie, Leipzig, Germany and CMC, Christian Medical College, Vellore, India.

*Pool 1, O26, O103, O111, O145, O157; pool 2, O55, O119, O125ac, O127, O128ab; pool 3, O86, O114, O126, O142. Intimin genes were amplified from bacterial cell extracts using universal intimin primers as described by Batchelor *et al.* (1999).
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