Identification of *Shigella* and Enteroinvasive *Escherichia coli* Strains by a Virulence-Specific, Monoclonal Antibody-Based Enzyme Immunoassay

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BALB/c mice were immunised with water extracts made from an *Escherichia coli* K-12 strain harbouring the shigella invasion plasmid, and hybridomas secreting antibodies specific to invasion plasmid-coded antigens were selected. On Western blots, antibodies produced by one of these clones (MAIC-1) recognised a protein of 43 kDa, which is the molecular mass of invasion plasmid coded antigen C (IpaC). When used in enzyme immunoassay against whole bacterial cells or against proteins secreted by actively growing bacteria, MAIC-1 clearly differentiated between invasive and non-invasive strains. Testing 123 enteroinvasive and 139 non-enteroinvasive strains the MAIC-1-based assay proved to be highly specific and sensitive in recognising enteroinvasive isolates. This test could be an inexpensive and rapid alternative to cumbersome virulence assays and a helpful technique in identifying *Shigella* or enteroinvasive *Escherichia coli* isolates.

The main etiologic agents of bacillary dysentery, still a major cause of diarrheal diseases in developing countries, are shigellae and enteroinvasive *Escherichia coli* (EIEC) (1). The members of the *Shigella* genus are the *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* strains, represented by 12, 13, 18 and 1 serotypes, respectively (2). EIEC strains belong to a restricted number of serogroups, most often expressing either O28ac, O29, O124, O136, O143, O144, O152, O164 or O167 O-antigenic specificities (3). Strains of EIEC express virulence factors identical to those of virulent *Shigella* strains; consequently, they are also able to infect, multiply within and spread to adjacent epithelial cells, which are the main steps of the pathogenesis of bacillary dysentery (4).

The traditional way to diagnose a *Shigella* infection is to isolate and identify the pathogen by biochemical and serological tests (3). Usually no confirmation of the isolated strain's virulence is required since all the members of the genus are considered pathogenic. Often, however, these methods are either slow, not sensitive enough or rely on the fast transport of the sample to the laboratory. Recently, several new assays became available to improve the detection of *Shigella* spp. With a few exceptions (5, 6), these techniques aim at demonstrating various DNA sequences located within different virulence-related genes of the pathogens. Various DNA hybridisation probes (7–9) as well as polymerase chain reaction (PCR) systems (10, 11) have been developed and successfully used for this purpose.

EIEC strains cannot be identified by traditional bacteriological techniques. Although the number of serotypes reported with enteroinvasive character are relatively low (3), one cannot rely solely on serotyping. Not all the strains with a particular O-antigen are invasive; moreover, virulent strains with O-antigens not recognised so far as one of the "potential EIEC-groups" might remain unnoticed. Although some features like lactose negativity, lysine decarboxylase negativity or the lack of motility are often recorded among EIEC isolates (12), these markers can not reliably differentiate EIEC from non-EIEC strains (13). Therefore, the virulent character of the suspected isolate

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should be confirmed. Although this can be done with the DNA probes or PCR systems developed to detect *Shigella* strains (7–9), these methods require technical facilities often not available in every laboratory. Alternatively, animal models (14) or tissue culture invasion tests (15) can be used to demonstrate the co-ordinated expression of the virulence factors. However, for time and cost reasons, these virulence assays are usually beyond the scope of the routine diagnostic laboratories.

Recently, using a rabbit immune serum raised against a virulent strain, and absorbed with an avirulent EIEC strain, we demonstrated by enzyme immunoassay (EIA) an antigenic moiety, a virulence marker antigen, shared by all the virulent *Shigella* and EIEC strains tested (16). The virulence marker antigen activity was coded by the invasion plasmid (Ip) (17) and was conferred predominantly by Ip-coded antigen C (IpaC) (18). Because the Ip-coded proteins are present in all the virulent *Shigella* and EIEC isolates, immunodetection of some of these antigens could be a simple approach in identifying these pathogens (13). Here we report on the development of such an EIA which uses an IpaC-specific

monoclonal antibody and which provides results within 24 hours.

Materials and Methods

Bacterial Strains. Escherichia coli K-12 strains (J53 and SP10) (19) were used to prepare the immunising antigen and to screen hybridomas. Strain SP10 harbours the 220 kilobase (kb) Ip of *Shigella* and expresses the Ipa-s (19). For the preliminary assays, invasive and non-invasive clone pairs of *Shigella flexneri* 5 (M90T and M90T-55) and *Shigella flexneri* 2a (YSH6000 and YSH6200) were provided by S.B. Formal (Walter Reed Army Institute of Research, Washington, DC, USA) and by C. Sasakawa (University of Tokyo, Tokyo, Japan), respectively. *Shigella flexneri* Y SFL1, EIEC O143 no. 2 and their avirulent derivatives, SFL 555 and no. 2/33, were described previously (20, 16). To test the specificity of the assay, *Shigella* and EIEC strains as well as several other pathogenic and non-pathogenic strains (Table 1) were obtained from S.B. Formal, L.R. Trabulsi (Escola Paulista Medicina, Sao Paolo, Brazil), P. Echeverria (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand), B.R. Davis (Centers for Disease Control, Atlanta, USA) and from several culture collections (the National Bacteriological Laboratory, Stockholm, Sweden; the Department of Clinical Bacteriology, Karolinska Institute, Stockholm, Sweden; and the De-

Table 1: Reactivity of monoclonal antibody MAIC-1 with enteroinvasive and non-enteroinvasive strains in enzyme immunoassay.

Genus/species	No. of strains tested	OD in EIA with colonies[a]		OD in EIA with streak[b]	
		Range	Average	Range	Average
Enteroinvasive[c]					
Shigella, virulent	83	0.011 – 2.260	1.076 ± 0.279	0.203 – 2.068	1.096 ± 0.291
EIEC, virulent	40	0.230 – 2.302	1.186 ± 0.327	0.269 – 2.210	1.237 ± 0.347
Subtotal	123	0.011 – 2.302	1.112 ± 0.300	0.203 – 2.210	1.142 ± 0.316
Non-enteroinvasive[d]					
Shigella, avirulent	11	0.007 – 0.080	0.022 ± 0.013	0.011 – 0.048	0.019 ± 0.011
EIEC, avirulent	7	0.000 – 0.052	0.020 ± 0.013	0.010 – 0.132	0.033 ± 0.043
ETEC	21	0.003 – 0.098	0.022 ± 0.014	0.002 – 0.055	0.020 ± 0.013
EPEC	4	0.002 – 0.067	0.020 ± 0.021	0.005 – 0.084	0.026 ± 0.038
EHEC	1	0.016 – 0.040	0.024 ± 0.010	0.016	–
Salmonella	22	0.005 – 0.109	0.022 ± 0.017	0.007 – 0.069	0.022 ± 0.015
Yersinia	6	0.011 – 0.040	0.021 ± 0.009	0.011 – 0.039	0.022 ± 0.012
Citrobacter	20	0.006 – 0.119	0.022 ± 0.017	0.008 – 0.084	0.025 ± 0.019
Enterobacter	12	0.012 – 0.114	0.044 ± 0.024	0.014 – 0.086	0.041 ± 0.022
Klebsiella	3	0.002 – 0.016	0.008 ± 0.004	0.012 – 0.024	0.016 ± 0.006
Morganella	10	0.005 – 0.137	0.025 ± 0.024	0.013 – 0.046	0.021 ± 0.009
Proteus	9	0.018 – 0.148	0.040 ± 0.024	0.019 – 0.092	0.039 ± 0.021
Plesiomonas	3	0.006 – 0.039	0.014 ± 0.009	0.007 – 0.017	0.011 ± 0.005
Pseudomonas	10	0.008 – 0.078	0.024 ± 0.015	0.008 – 0.073	0.021 ± 0.019
Subtotal	139	0.000 – 0.148	0.025 ± 0.019	0.002 – 0.132	0.025 ± 0.020

[a] Four individual colonies were used to inoculate the wells.
[b] A loopful taken from confluent growth was used to inoculate a well.
[c] Isolates from cases of bacillary dysentery, invasive for LLC-MK cells.
[d] *Shigella* or EIEC strains that had lost their invasive capability upon storage, non-enteropathogenic species and enteropathogenic species causing infections by mechanisms different from that of *Shigella*.
EIEC = Enteroinvasive *Escherichia coli*; ETEC = Enterotoxigenic *Escherichia coli*; EPEC = Enteropathogenic *Escherichia coli*; EHEC = Enterohemorrhagic *Escherichia coli*.

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For the different forms of EIA, bacteria were routinely grown on tryptic soy agar (TSA) (BBL, Becton Dickinson, USA) or in Luria broth supplemented with 45µg/ml lincomycin. Desoxycholate-citrate agar and brain heart infusion (BHI) were purchased from Oxoid, UK; Penassay broth was from Difco, USA; and tryptic soy broth (TSB) from BBL.

Antigen Preparation. Water extracts were prepared as described by Oaks et al. (21).

Monoclonal Antibodies. Male BALB/c mice were immunised intraperitoneally (i.p.) with approximately 0.1 mg water-extract protein of strain SP10 in complete Freund's adjuvant (Sigma, USA). The injections were repeated two weeks later, with the antigen suspended in incomplete Freund's adjuvant. After three weeks, the animals were boosted again with antigen dissolved in PBS. Three days after the last booster dose, the animals were sacrificed and spleen cells were fused with SP2/0 cells. Only those clones giving a positive reaction by EIA (absorbance $\geq$ 0.2) against water extract made from SP10, but not from J53, i.e. the plasmid-less parent strain were further investigated.

Hybridoma cells were routinely grown in RPMI 1640 medium (Gibco/Life Technologies, USA) containing 10 % heat inactivated fetal calf serum and supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin, all from Flow Laboratories (UK). For monoclonal antibody mass-production, the above medium was used without fetal calf serum but supplemented with non-essential amino acids (Flow Laboratories), 2.5 µg/ml transferrin and 2.5 µg/ml insulin (Sigma, USA).

The isotypes of the resulting MABs were determined by Ouchterlony immunodiffusion using rabbit anti-mouse immunoglobulins from Cappel/Organon Teknika (USA) and from Litton Bionetics (USA).

A *Salmonella* Re lipopolysaccharide (LPS)-specific MAB (MASM-2) and a MAB recognising *Shigella flexneri* 2a cell-wall antigen (MASF II:3,4) have been described previously (22, 23).

Enzyme Immune Assay (EIA). Hybridomas were screened using EIA plates sensitised with water extract. Flat bottom EIA plates (Costar, USA) were sensitised overnight at room temperature with 100 µl of water extract made from strains SP10 or J53 at a protein concentration of 25 µg/ml. Undiluted supernatants were tested against the two antigens using rabbit anti-mouse immunoglobulin, an alkaline-phosphatase conjugate (Dakopatts, Sweden).

EIAs were then conducted using plates sensitised with whole bacterial cells. One hundred µl of bacteria grown on TSA and suspended in bicarbonate buffer (pH 9.6) to OD$_{600}$ 0.2 were used to sensitise EIA plates overnight at room temperature, as described previously (24). Sensitised plates were blocked with 1 % bovine serum albumin (BSA) in PBS for 1 h at room temperature, and after washing 100 µl of MAB MAIC-1 diluted in PBS containing 0.05 % Tween and 0.1 % BSA was added. After 2 h of incubation at room temperature, anti-mouse IgG-HRPO conjugate (Jackson, USA) diluted 1:500 was added for 2 h. Reaction was developed after 20 minutes

with 0.1 mg/ml *o*-phenylenediamine dihydrochloride (Dakopatts), stopped with 2 N H$_2$SO$_4$ and the OD was measured at 492 nm.

Finally, EIAs were conducted using plates sensitised with actively growing bacteria. Colonies grown on desoxycholate-citrate plates were suspended in PBS up to a density of OD$_{600}$ 0.2. Wells of the EIA plates containing 100 µl Luria broth, TSB, BHI or Penassay broth were inoculated with 5 µl of these suspensions or were loop-inoculated from cultures grown on desoxycholate-citrate plates and incubated overnight at 37°C. All the liquid media were supplemented with 45 µg/ml lincomycin to suppress the growth of possible contaminants. Next day plates were reacted with MAIC-1 antibodies, and further processing of these plates was identical to that described above.

SDS-PAGE and Western Blot. Bacterial whole cell extracts were prepared, separated and blotted as described previously (18). Ip-coded antigens were detected on Western blots and identified, using a convalescent human serum, by their reported molecular weight (4) and by their absence in extracts made from a strain lacking the Ip.

Virulence Assays. In the guinea pig keratoconjunctivitis test, the method of Serény (14) was followed; whereas, the in vitro epithelial cell invasion assay was performed using LLC-MK cells (15).

Results

Production of Monoclonal Antibodies Specific to Virulent Shigella and EIEC. Immunisation with SP10 water extract yielded 13 hybridoma clones which reacted with SP10 water extract but not with J53 water extract in EIA. Two of the clones recognised an Ip-coded band corresponding to the molecular mass of IpaB (62 kDa) in Western blots (4). The remaining 11 clones gave strong signals with a band of approximately 43 kDa, the reported molecular mass of Ipa C (4). Since it has been shown that polyclonal antibodies recognising virulent *Shigella* and EIEC in EIA were specific to this antigen (18), clones of this latter group were further investigated. One of these clones, while negative against water extract of the non-invasive strain, gave higher readings in EIA (OD range: 0.480–0.540) than the other clones (OD range: 0.200–0.310) against water extract made from the invasive isolate (data not shown). This clone was named MAIC-1 and was selected for further studies. By immunodiffusion MAIC-1 was identified as subclass IgG$_1$. In Western blots, even at a dilution of 1:100, it strongly reacted with a band of 43 kDa present only in the invasive strain (Figure 1, lane A) but not in the Ip-less derivative (Figure 1, lane B). When undiluted, MAIC-1 also gave a weak signal with a band of

approximately 38 kDa in extracts of both the invasive and non-invasive clones (data not shown).

To test its reactivity against whole bacteria different dilutions of the serum-free MAIC-1 supernatant was assayed against three clone pairs of invasive and non-invasive strains. EIA plates were sensitised by decreasing number of whole bacterial cells suspended in coating buffer. As can be seen in Figure 2 (graph A), even undiluted supernatant did not react (absorbance < 0.1) with non-invasive strains when as much as 5 x 10^8 bacteria

A B

43 kDa — ■

Figure 1: Western blot with supernatant of monoclonal antibody MAIC-1 diluted 1:100. Lane A: extract of M90T; lane B: extract of M90T-55.

were used to sensitise the wells. On the other hand, 10^7 cells of the virulent strains gave absorbance values of 0.9 to 1.2 with the supernatant diluted 1:1280 (graph B). These results indicated that MAIC-1 was specific to clones harbouring the Ip of enteroinvasive strains and, therefore, was a suitable candidate in a monoclonal-based *Shigella*/EIEC-specific immunoassay.

Sensitisation of EIA Plates with Actively Growing Bacteria. Before testing MAIC-1 antibodies on a larger panel of isolates, attempts were made to shorten the required assay time. Recently, Andrews et al. (25) reported that during growth IpaB and IpaC were excreted into the culture medium. Therefore, we investigated whether sensitisation of EIA plates could be achieved by actively multiplying bacteria. While growing overnight in the wells of the EIA plates, bacteria sensitised the wells when an invasive strain (YSH6000), but not when its non-invasive derivative (YSH6200), was inoculated, irrespective of the medium used (Figure 3). Similar results were obtained when wells were infected with cells suspended in PBS or when they were loop-inoculated with single colonies from desoxycholate-citrate plates (data not shown). Further studies reported here were conducted with bacteria growing in Luria broth containing 45 µg/ml lincomycin.

Due to their unique surface characteristics, invasive strains are known to have higher surface hydrophobicity (26). We investigated whether the
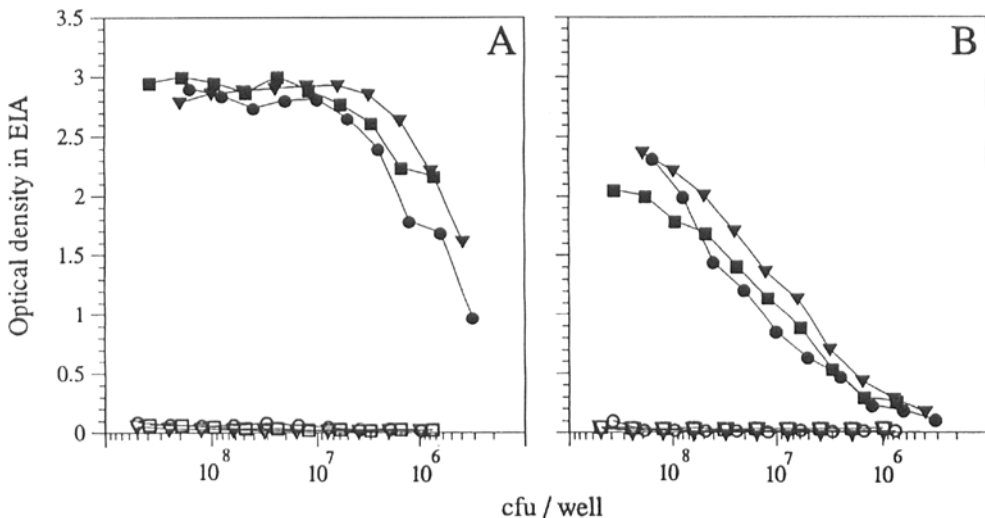

Figure 2: Reactivity of MAIC-1 against invasive strains and against their non-invasive derivatives in whole-cell enzyme immunoassay. A: undiluted MAB; B: MAB diluted 1/1280. Virulent strains: ● YSH6000 *Shigella flexneri* 2a; ■ M90T *Shigella flexneri* 5; and ▼ No. 2 *Escherichia coli* O143. Non-virulent strains: ○ YSH6200 *Shigella flexneri* 2a; □ M90T-55 *Shigella flexneri* 5; and ▽ No. 2/33 *Escherichia coli* O143

higher OD readings seen in wells, where invasive clones had been growing, were not due to the non-specific binding of the mouse immuno-globulins to some of the excreted/shedded components of the invasive strains. Serial dilutions of MAIC-1, those of a MAB specific to the *Shigella flexneri* 2a LPS (MASF II:3,4) (23) and those of one specific to *Salmonella* chemotype Re LPS (MASM-2) (22) were tested against *Shigella flexneri* 2a YSH6000 and YSH6200, respectively (Figure 4). As expected, MAIC-1 clearly differentiated between the invasive and non-invasive strains. On the contrary, the homologous *Shigella* LPS-specific antibody reacted equally well with both strains, while the *Salmonella*-specific one

Figure 3: Sensitisation of EIA plates by an invasive and a non-invasive strain growing in different media. ■ YSH6000 *Shigella flexneri* 2a, virulent; ❑ YSH6200 *Shigella flexneri* 2a, non-virulent. TSB: tryptic soy broth; LB: Luria broth; PA: Penassay broth; BHI: brain heart infusion.

gave no reaction with either strain. These results proved that MAIC-1 exhibited a specific reaction with Ip-containing strains, even when tested on plates sensitised with actively growing bacteria.

MAIC-1 EIA with Various Pathogenic and Non-Pathogenic Isolates. The specificity and sensitivity of the assay was tested on a broader scale. Virulent *Shigella* and EIEC strains; clones which had lost their invasive capability upon storage, as confirmed by the epithelial cell invasion assay (15) and by Serény test (14); apathogenic bacteria of other species; and bacteria causing enteric infections by various other mechanisms were investigated. *Shigella* strains represented 29 serotypes of the four species, while EIEC strains represented serogroups O28ac, O29, O124, O136, O143, O152, O164, O167. Strains were tested by inoculating four wells of the EIA plates with single colonies and the fifth one with a streak from confluent growth of the test bacteria from desoxycholate-citrate plates. The results are summarised in Table 1.

The average OD values of virulent enteroinvasive (*Shigella* and EIEC strains) colonies (1.112 ± 0.300) and streaks (1.142 ± 0.316) differed significantly (p < 0.01, unpaired t test) from the corresponding values of non-enteroinvasive colonies (0.025 ± 0.019, 0.025 ± 0.020, respectively). To evaluate the results for the individual strains, OD readings of colonies and streaks were divided into three categories: OD values above 0.212 (x - 3 SD of enteroinvasive colonies) were positive; those below 0.082 (x + 3 SD of non-enteroinvasive colonies) were negative; and those between 0.082 and 0.212 were intermediate. According to these cri-
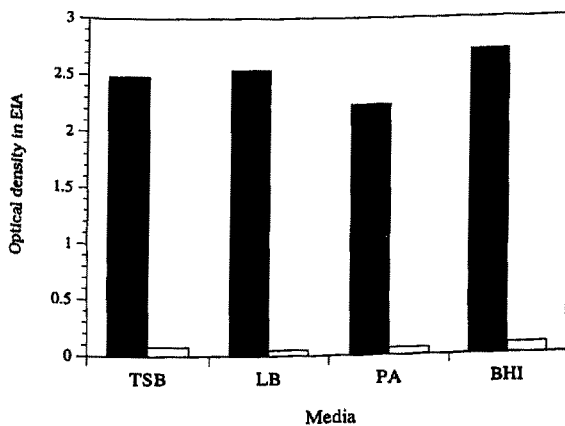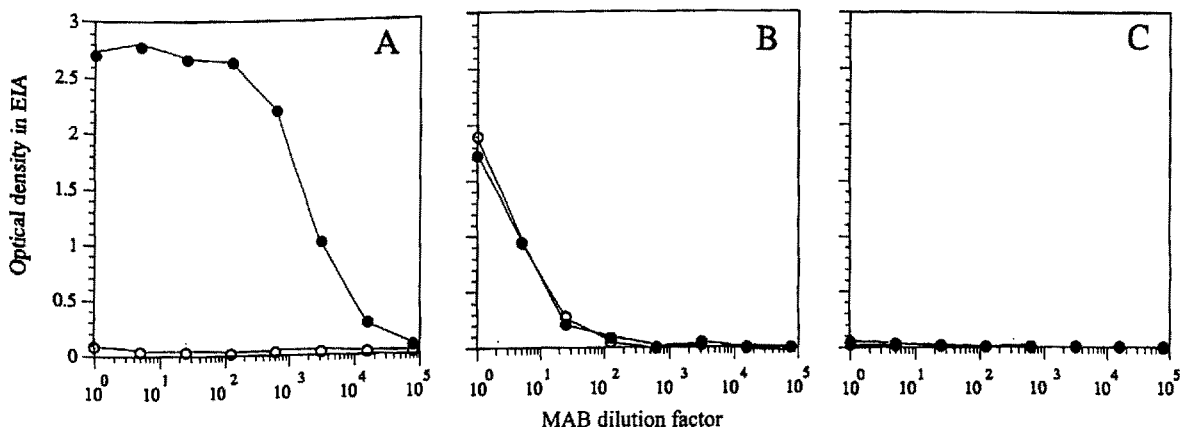

Figure 4: Reactivity of monoclonal antibodies MAIC-1, MASF II:3,4 and MASM-2 with a pair of invasive and non-invasive *Shigella* strains in EIA. ● YSH6000 *Shigella flexneri* 2a, virulent; ○ YSH6200 *Shigella flexneri* 2a, non-virulent. A: MAB MAIC-1; B: MAB MASF II:3,4; C: MAB MASM-2.

teria, out of the 615 tests (colonies or streaks) conducted with virulent (enteroinvasive) strains 608 were positive. Two tests with streaks fell into the intermediate category, and five colonies, provided by three *Shigella* strains (2, 2 and 1 colonies), were negative. It should be noted that the other colonies of these strains exhibited reactions clearly in the positive range. Of the 695 tests conducted with colonies or streaks of avirulent or non-enteroinvasive strains, none gave positive results, and only 18 (13 colonies and 5 streaks) fell into the intermediate class. It should be noted, however, that in these cases control wells without MAB gave readings within or close to the intermediate range (data not shown).

Discussion

The description of identical immunogenic proteins harboured by all virulent *Shigella* and EIEC strains suggested that identifying these pathogens by immunodetection could be feasible (16). This assumption was supported by our earlier experience using polyclonal absorbed sera specific to some of these proteins (13). The production of polyclonal antibodies is cumbersome, and due to the lot-to-lot variation, standardisation of such an immunoreagent is difficult to achieve. To overcome these problems, attempts were made to introduce monoclonal antibodies specific to enteroinvasive strains into the assay. By producing hybridomas specific to the water extract of an Ip-harbouring *Escherichia coli* K-12 strain, a clone (MAIC-1) was selected, and the resulting antibodies were tested against enteroinvasive and non-enteroinvasive strains by EIA.

On Western blots, MAIC-1 strongly labelled with band of approximately 43 kDa, the reported molecular mass of IpaC (4). We surmise that this band is indeed IpaC. This assumption was supported, beyond the identical MW values, by the facts that this band was missing from extracts of Ip-less strains (Figure 1), and, indirectly, that EIA results exhibited by this monoclonal antibody matched those observed with the mainly IpaC-specific, polyclonal, virulence marker antigen-specific serum (24). The 38 kDa band labelled weakly by the undiluted supernatant did not relate to the presence of the Ip (data not shown). Although the nature of this cross-reacting epitope is unknown, it did not cause any interference with the specificity of the EIA test, when water extract, whole bacterial cells or actively growing cells were used to sensitise the plates (Figures 2, 4).

Taking advantage of the fact that *Shigella* strains secrete IpaC during multiplication (25), the assay time was shortened to 24 h by eliminating the time required for cultivating bacteria for the whole-cell EIA (16). Inoculating the wells with individual colonies can be used for testing colonies suspect for *Shigella* (or EIEC) spotted on media (e.g. desoxycholate-citrate plates) used for primary isolation of this pathogen. Of the 492 colonies of virulent *Shigella*/EIEC strains, only five (from 3 strains) gave negative OD readings. Replicas of these colonies were tested for their ability to invade LLC-MK cells and were negative in the assay (data not shown). Therefore, these colonies were considered as clones which had lost the Ip upon cultivation on artificial media. It should be noted, however, that even these strains produced at least two positive colonies out of the four tested. Although data concerning the proportion of plasmid-less clones in the primary cultures of freshly isolated *Shigella* or EIEC strains were not available, we surmise that this proportion should be relatively low since the avirulent clones would be constantly counter-selected in the host. Similarly to EIA, losing the Ip would also cause negative results with Ip-specific DNA-based assays. This false-negative diagnosis could be overcome by testing sufficiently large number of colonies from clinical isolates.

Strains were also tested by inoculating wells from a mass of confluent bacterial growth. In this way, no virulent cultures gave negative results, and only two of the 123 virulent cultures exhibited intermediate readings. One of these strains produced two negative colonies, suggesting that this strain has a unique tendency to lose the Ip or the expression of IpaC. Using this mode of inoculation, the method could replace cumbersome virulence assays to investigate whether a particular *Escherichia coli* isolate expressing O-antigens characteristic of EIEC is indeed enteroinvasive.

It is noteworthy that the assay was not only sensitive but highly specific; non-invasive clones gave no positive results. The few intermediate readings, occurring with both colony and streak tests, were always accompanied with the aspecific binding of the conjugate by the bacteria (data not shown). This emphasises that all clones should also be tested against the conjugate and that clones giving OD values in the intermediate range should be retested to exclude this possibility. However, this does not seem to be a frequent phenomenon (15 of the 834 tests with non-enteroinvasive clones), and it has never been observed with *Shigella* or EIEC strains.

Based on our results, we propose that the EIA using MAB MAIC-1 could be a helpful tool in the bacteriological diagnostics of bacillary dysentery. The assay can either be used to test colonies suspected for *Shigella* or EIEC or to confirm the enteroinvasive character of isolated strains. In this way, virulence related antigen-specific immune assays could become simple alternatives for, or supplementary methods to, DNA based diagnostic techniques.

Acknowledgement

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