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# Egg Yolk Anti-BfpA Antibodies as a Tool for Recognizing and Identifying Enteropathogenic *Escherichia coli*

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## Abstract

Enteropathogenic *Escherichia coli* (EPEC) is a major aetiological agent of childhood diarrhoea in developing countries. The structural repeating protein A subunit, BfpA, found in the bundle-forming pilus, is one of the virulent factors for EPEC pathogenesis. Recombinant BfpA in laying hens elicited sustained and vigorous antibody production. Immunoglobulin Y (IgY) anti-BfpA antibodies were recovered from egg yolk, purified and characterized. Immunoadsorption with whole extracts of the isogenic *E. coli* EPEC adherence factor (EAF) strain that lacks BfpA rendered the resulting IgY preparations capable of: (a) recognizing purified or recombinant BfpA proteins in a dose-dependent fashion; (b) blocking the colonization of HeLa cells by EPEC EAF<sup>+</sup>, *in vitro*; (c) specifically identifying *E. coli* bearing EAF<sup>+</sup>; and (d) inhibiting the growth of *E. coli* EAF<sup>+</sup> but not the EAF strain. IgY anti-BfpA is potentially useful as a specific, low-cost immunobiological reagent to screen human faecal specimens for the presence of EPEC.

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Received 24 September 2002; Accepted in revised form 10 January 2003

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## Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of diarrhoea amongst infants in developing countries [1–6]. Diarrhoea caused by EPEC is often more severe than that caused by other pathogens [7, 8] and is frequently associated with concurrent nutritional complications [9]. Furthermore, mortality rates because of EPEC diarrhoea tend to be high, with dramatic numbers of over 30% reported for selected outbreaks [8]. In Brazil, EPEC is the leading cause of childhood diarrhoea in the major urban centres [4–6], and the resulting disease is usually very severe, accounting for the great majority of cases visiting emergency rooms [5]. The initial phase of an EPEC infection is characterized by the colonization of intestinal cells by clusters of bacteria [10, 11]. This pattern of colonization, called localized adherence (LA) [12–14], is associated with symptomatic disease and is dependent on the presence of a 50–70 MDa plasmid, referred to as the EPEC adherence factor (EAF) plasmid [15, 16], which is most commonly found amongst those EPEC strains asso-

ciated with outbreaks [17]. Strains cured of the EAF plasmid no longer display LA *in vitro* and are less virulent in human volunteers [17]. The colonization factor responsible for LA, called bundle-forming pilus (BFP), was identified [18, 19] and the gene for its repeating structural subunit (BfpA, pilin A and bundlin) cloned [20, 21]. The gene *bfpA* is located on the EAF plasmid, and transposon-mediated disruption of this gene abrogates LA [20]. Most importantly, mature BFP containing BfpA is inducibly expressed on the outer membrane of EPEC [19]. Antisera containing specific anti-BfpA antibodies markedly inhibit LA [18, 22, 23], lending support to their use as a tool to detect EPEC. Hens hyperimmunized with venoms of the Brazilian snakes of the genus *Bothrops* and *Crotalus* produced antibodies capable of recognizing, combining with and neutralizing the toxic and lethal components of the venoms [24]. We, therefore, decided to develop a process for the preparation of anti-BfpA immunoglobulin Y (IgY) antibodies using recombinant BfpA as an immunogen and using adult laying hens. Here, we describe the methods

used to prepare, analyse and standardize anti-BfpA IgY preparations that block localized EPEC adherence to HeLa cells and bacterial growth *in vitro*.

## Material and methods

**Cell line and bacterial hosts.** HeLa cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 U/ml of penicillin/streptomycin. B171 is an EAF(+) (O111:NM) EPEC strain; B171-4 is a B171 EAF-cured derivative. All bacterial strains were maintained at  $-70^{\circ}\text{C}$  or in stabs and slants of Luria Bertani (LB) agar, for long- and short-term storage, respectively, and propagated in liquid LB broth supplemented with appropriate antibiotics when required. Bacterial chemical transformations were carried out by the  $\text{CaCl}_2$  method described elsewhere [25].

**DNA preparation and manipulation.** EAF plasmid DNA was extracted from an overnight suspension of EAF(+) EPEC strain B171, using the QIAGEN Plasmid Kit (QIAGEN Inc., Valencia, CA, USA), following the manufacturer's instructions. Plasmids used as cloning and expression vectors were extracted from their respective hosts by the alkaline lysis method described elsewhere [25]. Restriction enzyme digestions, polymerase chain reaction (PCR) amplifications and DNA ligations employed enzymes and buffers obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA) and Pharmacia Biotech (Uppsala, Sweden) and followed standard protocols [25].

**Expression and purification of recombinant BfpA.** Recombinant BfpA was expressed in *E. coli* using conventional methods and is described elsewhere [26]. Briefly, forward and reverse primers, 32 bp in length, corresponding to the 5' and 3' ends of *bfpA* (see PCR validation method below), were designed based on published sequences (GenBank accession number Z12295) and used to amplify the mature coding sequence by PCR, using total genomic DNA as a template, isolated from the EPEC EAF<sup>+</sup> strain B171. Amplified DNA was cloned in frame into a pQE-30 vector (QIAGEN Inc.) that possesses a 6x-*His*-tag-coding  $\text{NH}_2$ -leading sequence, generating the construct termed pEU84. The identity of the cloned insert was validated by partial sequencing and restriction-enzyme mapping. pEU84 was used to transform *E. coli* DH5- $\alpha$  or M15 (pREP4) strains, and positive clones were selected by growth on ampicillin/kanamycin-containing agar plates. Induction of synthesis of recombinant 6x-*His*-tag fusion BfpA polypeptide was carried out by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (Promega, Madison, WI, USA) at 0.4 mM and purification of recombinant protein by *His*-binding nickel agarose affinity batch chromatography, carried out essentially as recommended by the manufacturer (QIAGEN

Inc.), using cleared bacterial lysates made in 8 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$ /0.01 M Tris-HCl, pH 8.0. Eluates (8 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$ /0.01 M Tris-HCl, pH 4.5) were dialysed extensively against distilled water, then against 50 mM Tris-HCl, pH 7.4/100 mM NaCl, dried down and reconstituted in Milli-Q water.

**Bacterial extracts.** *E. coli* strains were grown in LB medium supplemented with the appropriate antibiotics at  $37^{\circ}\text{C}$  until the optical density at 600 nm reached 0.4–0.6. Bacteria were collected in fresh medium and incubated at  $37^{\circ}\text{C}$  under inducing conditions for up to 3 h. Induced bacteria were collected by centrifugation and suspended in distilled water. After addition of two volumes of Laemmli's sample buffer, the suspension was heated at  $95^{\circ}\text{C}$  for 5 min. The extracts were kept at  $-20^{\circ}\text{C}$ .

**Animals.** Three groups of 12 hens, aged around 6 months, weighing about 1.7 kg, in good health and laying five to six eggs per week, were used to produce anti-BfpA IgY antibodies. The animals were kept in cages ( $25 \times 40$  cm) and were provided *ad libitum* with proper food and water and housed in standard installations. Prior to selection for these experiments, the young chickens were vaccinated against the infectious agents causing poultry diseases in Brazil. All animals were maintained according to the Animal Welfare International Recommendations [27].

**Immunization.** The immunization schedule used was essentially as described [24]. After a preliminary trail to define the immunization schedule, groups of 12 hens were primed on day 0 by intramuscular injection of 100  $\mu\text{g}$  of recombinant BfpA incorporated into complete Freund's adjuvant (CFA). Boosters were applied 21, 50, 65 and 80 days later with the same doses of BfpA, the first two being incorporated into incomplete Freund's adjuvant and the other only into saline. Blood samples and eggs were collected immediately before and during the course of immunization. Blood samples were allowed to clot overnight at  $4^{\circ}\text{C}$  and the separated sera stored at  $-20^{\circ}\text{C}$ . The eggs were stored at  $4^{\circ}\text{C}$  before used to prepare IgY.

**Purification of chicken IgY.** The method used to obtain crude preparations of yolk IgY was essentially as described [24]. In brief, one volume of whole egg yolk was dissolved in 10 volumes of distilled water, the pH was adjusted to 5 with HCl 6 N and incubated at  $4^{\circ}\text{C}$  for 14–16 h, followed by centrifugation at  $10,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The supernatant was collected and precipitated with 19% of sodium sulfate (w/v) under agitation for 2 h and then centrifuged at  $10,000 \times g$  for 30 min at room temperature. The precipitated material was suspended in water and dialysed against 25 mM sodium phosphate, pH 8.0.

Ten millilitre samples of this IgY preparation (190 mg of total proteins) were applied to a Q-Sepharose FF (Amersham Pharmacia, Biotech AB, Uppsala, Sweden) column previously equilibrated with 25 mM sodium phosphate, pH 8.0. The column was washed with the above

buffer (starting buffer). Proteins were eluted with a linear NaCl gradient, beginning with starting buffer and reaching a limit of 500 mM NaCl. Anti-BfpA antibodies, assayed by the enzyme-linked immunosorbent assay (ELISA) method [28], emerged with the main protein peak at about 55 mM. Fractions containing anti-BfpA antibodies were pooled together, concentrated and dialysed against 0.15 M NaCl, and the protein contents were determined. Protein concentration was determined by previously described methods [29, 30]. The IgY anti-BfpA preparations contained 12–15 mg of total protein/ml. The presence of IgY was verified by Western blot using a sheep peroxidase conjugate anti-IgY IgG (Calbiochem–Novabiochem Corporation, La Jolla, CA, USA) as a secondary antibody. The purified IgY preparations were stored in 1.5 M NaCl at 4 °C.

**Stool specimens.** Children under 2 years of age, visiting the 'Emergência e Enfermaria', Hospital Ferreira Machado, Campos dos Goytacazes, RJ, Brazil, with acute or persistent diarrhoea, and whose guardian consented to their participation in the study, were selected as stool donors. Diarrhoea diagnosis was based on acute and persistent watery diarrhoea, fever and dehydration. Upon collection, the stool specimens were alternatively cultured or stored at 4 °C. Stool samples, freshly collected or previously frozen, were cultured in pre-enriched fast green broth liquid medium (Merck, Darmstadt, Germany) to grow *E. coli* for 24 h at 37 °C. Samples were transferred to selective solid eosin methylene blue medium. Colonies exhibiting fast green *E. coli* phenotype were recovered and reconstituted in phosphate-buffered saline, pH 7.4. The presence of EPEC was determined by the agglutination test using specific rabbit antibodies to the various EPEC serogroups: polyvalent serum A – serogroups *E. coli* O26, O55, O111 and O119; polyvalent serum B – serogroups *E. coli* O114, O125, O142 and O158; polyvalent serum C – O86, O126, O127 and O128 (KIT, Probac do Brazil, São Paulo, SP, Brazil). Twelve stool specimens exhibiting the highest agglutinating titres with these sera were selected and included in the experiments. The specimens exhibiting EPEC antigens were expanded first in LB and later in DMEM (Gibco BRL, Rockville, MD, USA), to induce the expression of BfpA. EPEC B171/EAF(+) and EPEC(-) were used as a positive control. The bacterial suspensions were centrifuged for 20 min at 7 000 × *g* at 4 °C, the supernatants discarded and the bacterial mass used as intact cells for dot-immunobinding assay [31] or as bacterial cell extracts for Western blotting [32], DNA preparation or adsorption assays.

**Localized cell adherence assay.** HeLa cell monolayers plated onto tissue culture plates were maintained in complete DMEM supplemented with 10% FCS and 10 U/ml of penicillin/streptomycin, until cells reached 25–50% confluency. After washing the cells three times with sterile 0.15 M NaCl, 300 µl of antibiotic-free DMEM was added.

To one group of plates was added 0.15 M NaCl (cell controls); to a second was added 10 µl of an overnight suspension of EPEC B171; and, to the third and fourth groups was added also 10 µl of an overnight suspension of EPEC B171 but previously treated with different concentrations of nonimmune IgY and IgY anti-BfpA, respectively. Plates were incubated at 37 °C under 5% CO<sub>2</sub> atmosphere for 3 h, washed three times with 0.15 M NaCl, methanol fixed and Giemsa stained for microscopic evaluation.

**Polymerase chain reaction analysis of stool specimens.** PCR amplification of virulence gene factors was carried out using the following primers, designed from published sequences: *bfpA* (GenBank accession number P33553): P1 – 5'-ACT ATG GAT CCC TGT CTT TGA TTG AAT CTG CA; P2 – 5'-ATA TTA AGC TTT TAC TTC ATA AAA TAT GTA AC; *espB* (GenBank accession number AF144010): P3 – 5'-ACT ATG GAT CCA ATA CTA TTG ATT ATA CTA AT; P4 – 5'-ATA TTA AGC TTT TAC CCA GCT AAG CGA ACC GAT TG; and *intimin* (*eaeA*, GenBank accession number ECU59504): P5 – 5'-ACT ATG GAT CCA TTA CTC ATG GTT TTT ATG CC; P6 – 5'-ATA TTA AGC TTT TAT TTT ACA CAA ACA GAA AAA GC. Control DNA templates were prepared from EPEC reference strains: B171 (EAF<sup>+</sup>, O111:NM) and B171-4 (isogenic EAF<sup>-</sup>) [33, 34]. As extensive result variation is observed with a given method of DNA preparation, DNA from consistently negative specimens was prepared by the Tris-HCL EDTA Licl TritonX (TELT) method [33, 34], which uses 50 mM Tris-HCL, pH 8.0, buffer containing 62.5 mM ethylenediaminetetraacetic acid, pH 9.0/2.5 M LiCl and 4% v/v Triton X-100 as lysis solution. Culture-enriched negative faecal samples were also incubated overnight in 5 ml LB broth. The conditions for DNA amplification were set for a 50 µl reaction containing 10–20 ng of DNA template and 50 pmol of each forward and reverse primers, as follows: 95 °C, 5 min (one cycle); 94 °C, 1 min; 47 °C, 2 min; 72 °C, 2 min (30 cycles); 72 °C, 15 min (one cycle). Amplified fragments were analysed by electrophoresis in ethidium bromide-containing 0.8% agarose gels.

**Immunochemical methods.** ELISA [28], dot-immunobinding assay [31], SDS-PAGE and Western blotting were performed as described elsewhere [32] and previously. In ELISA, Western blotting and dot-immunobinding assay [31], a sheep peroxidase conjugate anti-IgY IgG (Calbiochem–Novabiochem Corporation) as the second antibody was used.

## Results

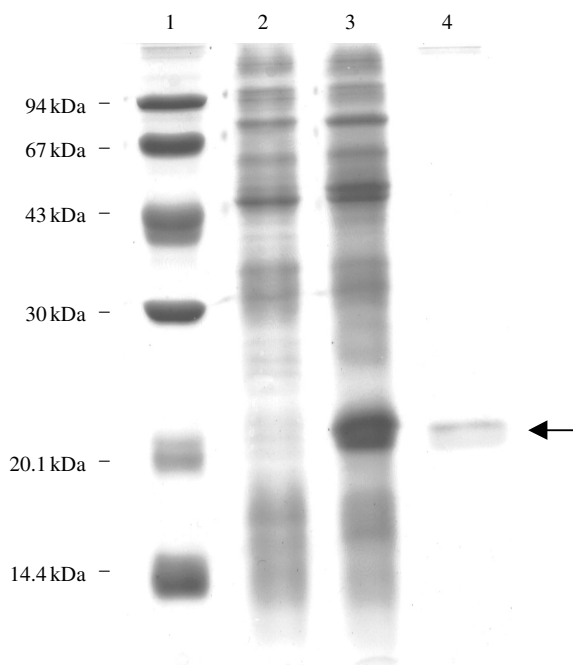
### Expression and purification of recombinant BfpA

*E. coli* DH5- $\alpha$  strain transformed with the plasmid construct pEU84, bearing the coding region of mature BfpA,

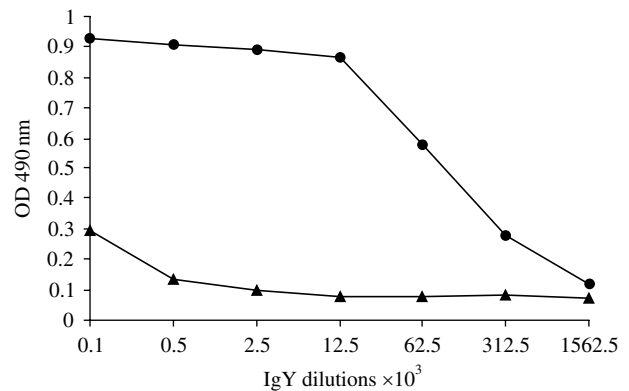
was grown in LB medium supplemented with the appropriate antibiotics at 37 °C, under shaking and aeration. Induction of synthesis of recombinant 6x-His-tag fusion BfpA polypeptide was carried out by the addition of IPTG, at 0.4 mM. Bacterial extract samples were subjected to SDS-PAGE under reducing conditions. A 20.1 kDa protein band appeared on the induced but not in noninduced bacteria (Fig. 1, lanes 2 and 3). A single protein band of 20.1 kDa was eluted when induced *E. coli* DH5- $\alpha$  extracts were fractionated by batch affinity chromatography on His-tag-binding nickel agarose (Ni-NTA agarose) (Fig. 1, lane 4).

### Antibody production

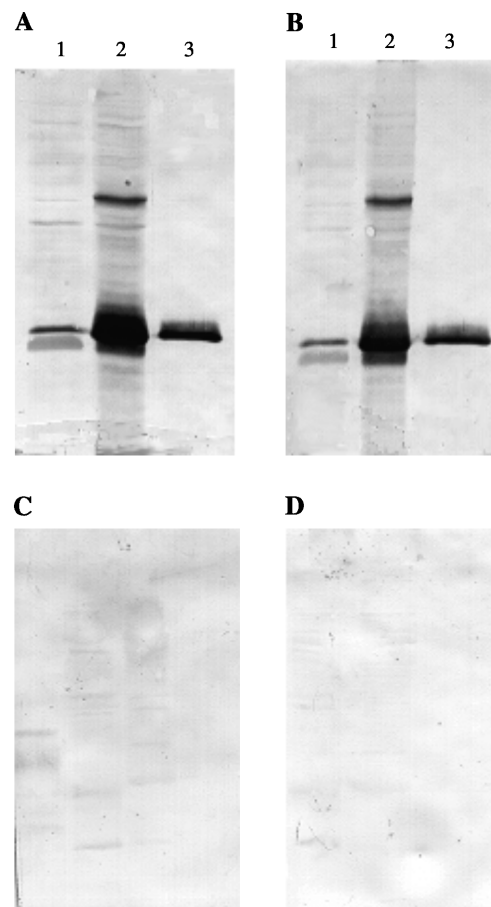
Laying hens were primed with purified recombinant BfpA (100  $\mu$ g in CFA) and boosted consecutively on the days 21, 50, 65 and 80 after priming with equal amount of the antigen, the first two injections with BfpA incorporated



**Figure 1** Expression and purification of recombinant BfpA. *Escherichia coli* DH5- $\alpha$  strain transformed with the construct pEU84 was grown in Luria Bertani medium supplemented with the appropriate antibiotics at 37 °C, under shaking and aeration. Induction of synthesis of recombinant 6x-His-tag fusion BfpA polypeptide was carried out by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at 0.4 mM and purification of recombinant protein by His-binding nickel agarose affinity batch chromatography on His-binding nickel agarose affinity (Ni-NTA agarose) batch chromatography. Bacterial extract samples were subjected to 15% SDS-PAGE under reducing conditions and stained with Coomassie blue. Lane 1, Mr (molecular weight) (kDa); lane 2, noninduced *E. coli* DH5- $\alpha$ ; lane 3, IPTG in induced *E. coli* DH5- $\alpha$ ; lane 4, recombinant BfpA purified on Ni-NTA agarose chromatography. ( $\blackleftarrow$ ), BfpA position.



**Figure 2** Enzyme-linked immunosorbent assay of egg yolk immunoglobulin Y (IgY) anti-BfpA. IgY (15.0 mg/ml) was purified from eggs yolk before immunization ( $\blacktriangle$ ) or 90 days after immunization of hens with recombinant BfpA ( $\bullet$ ). Recombinant BfpA was used as the antigen. OD, optical density.



**Figure 3** Western blot analysis of *Escherichia coli* DH5- $\alpha$  transfected with *bfpA* gene. Lane 1, noninduced *E. coli*; lane 2, isopropyl- $\beta$ -D-thiogalactopyranoside-induced *E. coli*; lane 3, purified BfpA. The blotting was revealed with serum from hens immunized with BfpA (A), purified egg yolk immunoglobulin Y (IgY) anti-BfpA (B) and serum or egg yolk IgY obtained and purified before immunization with BfpA, respectively (C and D).

into incomplete Freund's adjuvant and the others diluted in saline, produced specific anti-BfpA antibodies as assayed by the ELISA method. Anti-BfpA antibodies appeared in the serum in the first weeks after immunization. The antibody titres increased rapidly, attaining a plateau around 75 days later and remaining at high titres over 6 months (data not shown). These antibodies were transferred to and concentrated in the egg yolks.

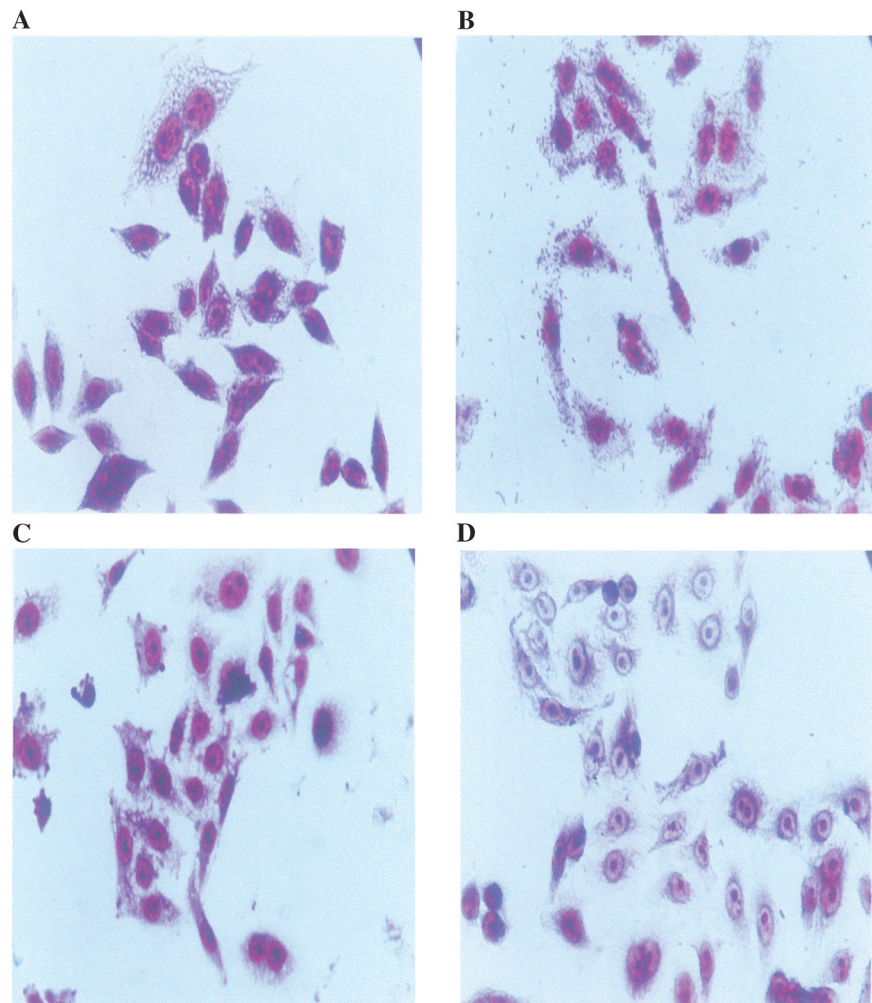
#### IgY anti-BfpA purification

IgY anti-BfpA antibodies were purified from egg yolk by positive precipitation with 19% saturation of sodium sulfate, followed by Q-Sepharose FF column chromatography. Figure 2 shows that the egg yolk-purified IgY contained high anti-BfpA antibody titres, as assayed by ELISA. IgY present in these preparations exhibited the typical L and H chains, as detected both by SDS-PAGE under reduction conditions and by Western blot analyses (data not shown). Purified IgY anti-BfpA preparations

were also capable of identifying BfpA either recombinant or in whole-cell protein extracts of *E. coli* bearing the plasmid pEU84 (DH5- $\alpha^+$ ) but not in *E. coli* free of the plasmid pEU84 (DH5- $\alpha^-$ ). Figure 3 shows both serum and egg yolk IgY preparations from the immunized hens (Fig. 3A,B), IgY obtained before immunization (Fig. 3C,D) recognized a 20.1 kDa protein band in the extracts of noninduced *E. coli* DH5- $\alpha(+)$  (Fig. 3A,B, lane 1). This band was strongly expressed by induced DH5- $\alpha(+)$  (Fig. 3A,B, lane 2) and exhibited an electrophoretic profile similar to that of the purified recombinant BfpA (Fig. 3A,B, lane 3).

#### IgY anti-BfpA inhibits in vitro adherence of EPEC on HeLa cells

EPEC samples preincubated with IgY anti-BfpA lost their ability to adhere on HeLa cells, *in vitro* (Fig. 4C,D). In contrast, under similar conditions, EPEC samples incubated with IgY from preimmunized hens adhered and formed typical LA on these cells (Fig. 4B). HeLa cells



**Figure 4** Inhibition of enteropathogenic *Escherichia coli* (EPEC)-localized adhesion on HeLa cells by purified immunoglobulin Y (IgY) anti-BfpA. A, cells incubated with Dulbecco's modified Eagle's medium; B, cells incubated with EPEC (B171/EPEC adherence factor (EAF)<sup>+</sup>) preincubated with 800 µg of egg yolk IgY from hens before immunization; C and D, cells incubated with EPEC EAF(+) pretreated with 400 µg and 800 µg of purified IgY from hens immunized with recombinant BfpA, respectively.

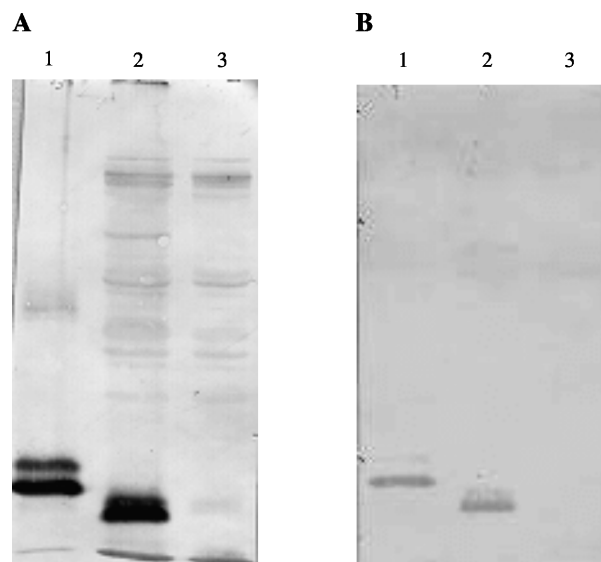
incubated with medium showed normal morphology (Fig. 4A).

#### Pre-adsorption of anti-BfpA with *E. coli* EAF(-)/(+)

Samples of egg yolk IgY anti-BfpA preparations were extensively pre-adsorbed with *E. coli* EAF(+) or with *E. coli* EAF(-) and analysed by Western blotting or by ELISA. Figure 5 shows that samples of IgY anti-BfpA pre-adsorbed with *E. coli* EAF(+) lost their ability to recognize recombinant BfpA or native BfpA in whole extracts of *E. coli* EAF(+) (Fig. 5B). In contrast, IgY anti-BfpA pre-adsorbed with *E. coli* EAF(-) retained their ability to recognize BfpA (Fig. 5A).

#### Recognition of EPEC in stool specimens by IgY anti-BfpA

The specificity of the pre-adsorbed IgY anti-BfpA to detect *E. coli* bearing BfpA was tested on *E. coli* samples isolated from stool specimens obtained from children under 2 years of age with diarrhoea and compared with that of EPEC EAF(+) (positive control) or EPEC EAF(-) (negative control). The stool specimens were EPEC positive by agglutination test (Table 1) using the polyvalent antisera A, B and C. Whole *E. coli* bacterial extracts or DNA from the expanded stool specimens were analysed by Western blotting using pre-adsorbed IgY anti-BfpA (0.13 µg/ml) as the first antibody (Fig. 6A), or by PCR analysis using *bfpA*



**Figure 5** Western blot analysis of pre-adsorbed purified egg yolk immunoglobulin Y (IgY) anti-BfpA. Samples of IgY anti-BfpA were adsorbed with enteropathogenic *Escherichia coli* (EPEC)(-)/B171 (A) or with EPEC adherence factor (EAF) (+)/B171 (B) and used to reveal the transferred protein bands from SDS-PAGE to nitrocellulose membranes as indicated: lane 1, purified BfpA; lane 2, *E. coli* EAF(+); lane 3, *E. coli* EAF(-).

**Table 1** *Escherichia coli* agglutination by polyvalent rabbit sera

Stool specimens	Antiserum A*	Antiserum B†	Antiserum C‡
1	+	+	+
2	-	+	-
3	-	+	-
4	-	+	+
5	-	-	+
6	-	+	-
7	+	+	-
8	+	+	-
9	+	+	+
10	+	+	+
11	+	+	+
12	+	-	+

-, no agglutination; +, agglutination to a different degree.

\*Serogroups O26, O55, O111 and O119.

†Serogroups O114, O125, O142 and O158.

‡Serogroups O86, O126, O127 and O128.

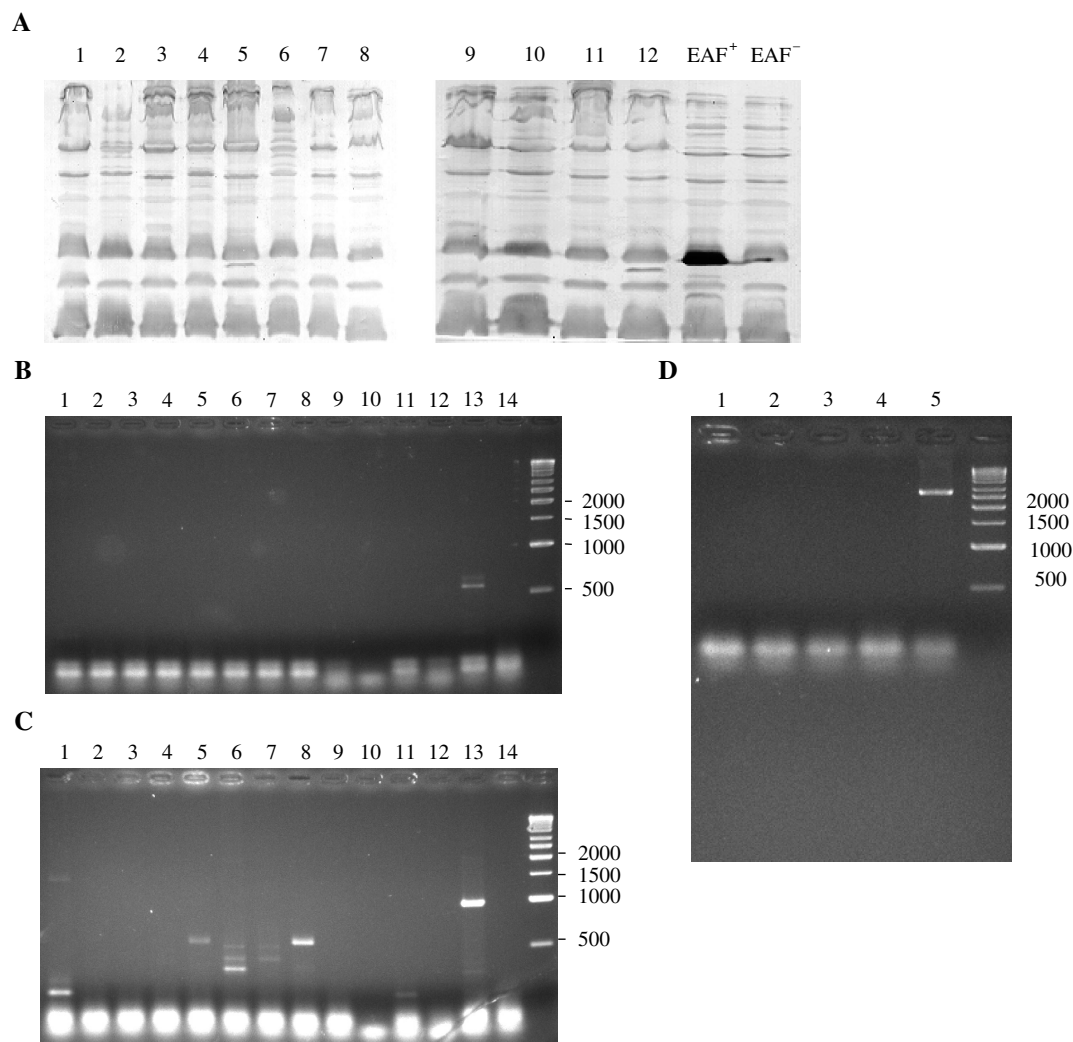
or *espB* primers (Fig. 6B,C), respectively. To plate A, bacterial protein extracts from the 12 stool specimens (wells 1–12) or those from *E. coli* EAF(+) or EAF(-) were applied. To plates B and C, DNA from the corresponding stool specimens (wells 1–12) used in plate A was applied; to wells 13 and 14, DNA of EPEC EAF(+) (positive control) and DNA from *Staphylococcus aureus* (negative control), respectively, was applied. The presence of the *intimin* gene was also investigated (Fig. 6D) using specific primer and DNA from the stool specimens 5, 6, 7 and 8 (wells 1–4) and DNA of EPEC B171/EAF(+) (well 5). The following findings were observed: (a) BfpA protein was clearly detected by IgY anti-BfpA in EAF(+) (Fig. 6A); (b) *bfpA* gene was detected only in DNA from EAF(+) sample (Fig. 6B); (c) the gene *espB* was detected in DNA from EAF(+) and, although with different electrophoretic mobilities, also in DNA from the stool specimens 5, 6 and 8; (d) *intimin* gene was detected only in EAF(+).

#### IgY anti-BfpA inhibits *in vitro* growth of EPEC

The pre-adsorbed IgY anti-BfpA inhibits, *in vitro*, the growth of *E. coli* EAF(+) but not of *E. coli* EAF(-) (Fig. 7).

#### Discussion

EPEC is a leading cause of community and nosocomial forms of diarrhoea in developing countries [35, 36]. Measures aiming to prevent, treat and control the infections involve permanent and large-scale epidemiological surveillance. To accomplish these objectives, specific immunogens, adequate animals and reliable methods to obtain and prepare the antibodies are needed. Availability of specific and low-cost immunobiological reagents is therefore an



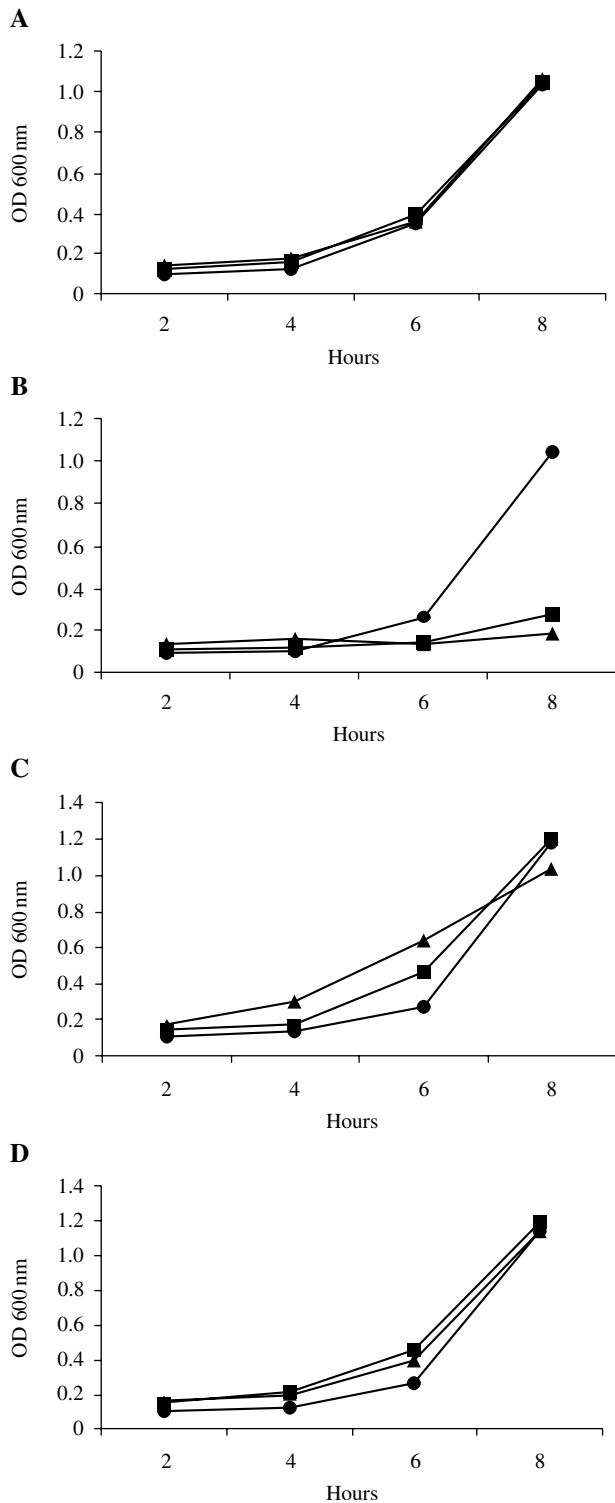
**Figure 6** Specificity of the immunoglobulin Y (IgY) anti-BfpA to detect *Escherichia coli* bearing BfpA. *E. coli* isolated from 12 stool specimens obtained from children of less than 5 years of age with diarrhoea were compared with enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF)(+) (positive control) or EPEC EAF(-) (negative control). Whole *E. coli* protein extracts were analysed by Western blotting using the pre-adsorbed IgY anti-BfpA (0.13 µg/ml) as the first antibody (A). DNA preparations from *E. coli* stool specimens were analysed by polymerase chain reaction using *bfpA* and *espB* as the probe to detect *bfpA* and *espB* genes, respectively (B and C). Wells 1–12, DNA from *E. coli* of stool specimens; well 13, DNA of EPEC EAF(+) (positive control); well 14, DNA of *Staphylococcus aureus* (negative control). *intimin* gene was detected using DNA from the stool specimens 5, 6, 7 and 8 and from the DNA of EPEC B171/EAF(+) positive (control) (D). Size markers are given on the right hand side.

absolute requirement. This study describes the method used to prepare specific chicken IgY antibodies endowed with the ability to recognize *E. coli* pathogenic strains expressing BfpA. This protein, as a structural subunit of the BFP, is at least one of the mediators involved in the attachment of *E. coli* on enterocytes [18, 19], a phenomenon markedly blocked by antisera containing specific anti-BfpA antibodies [18, 22].

Early in the EPEC infection, the bacteria adhere to microvilli and to each other probably by virtue of BFP [10, 11]. BfpA is located on the EAF plasmid, and transposon-mediated disruption of this gene abrogates LA [20]. Native BFP is expressed on the outer membrane of EPEC [19]. Most importantly, SL3261 (pBfpA) orally administered

to BALB/c mice was capable of eliciting a sustained and vigorous humoral immune response to BfpA [22].

Chickens were selected as the animal to produce anti-BfpA antibodies for several reasons: First, hens hyperimmunized with venoms of the Brazilian snake of the genus *Bothrops* and *Crotalus* produced antibodies capable of recognizing, combining and neutralizing the toxic and lethal actions of snake venoms [24]. These antibodies are also continuously transferred and concentrated into the egg yolks, mostly as IgY, from which they can be easily isolated [37–40]. Second, as a laying hen produces approximately 20 eggs per month, over 2 g of IgY per month can be isolated. The IgY concentration in chicken serum is approximately 5–7 mg/ml [37–40], therefore 2 g



**Figure 7** Culture growth of enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF)(+) or EAF(-) in Dulbecco's modified Eagle's medium (DMEM) in the presence of immunoglobulin Y (IgY) anti-BfpA pre-adsorbed with EPEC EAF(+) or EAF(-). A and B, EPEC EAF(+); C and D, *Escherichia coli* EPEC EAF(-); A and C, *E. coli* culture supplemented with IgY anti-BfpA adsorbed with EPEC EAF(+); B and D, *E. coli* culture in DMEM plus IgY anti-BfpA adsorbed with EPEC EAF(-).

of egg IgY correspond approximately to the IgY content of 300 ml of serum or 600 ml of blood. Only large mammals can produce comparable amounts of serum antibodies [37–40]. Third, hens can be used for antibody production throughout the entire egg-laying period [37–40]. Chicken antibodies can be used in many immunological assays without interference caused by human complement system or rheumatoid factors [37–40]. In addition, antibody collection from eggs conforms to the animal welfare requirements for several reasons: the animals, instead of being subjected to vein punctures or even killed for blood collection, are maintained under the best conditions of housing and food supply [37–40].

The methodology reported herein describes the preparation of anti-BfpA IgY antibodies endowed with the ability to recognize *E. coli* bearing BfpA. The immunization schedule, priming the hens with BfpA incorporated in CFA and subsequently boosted with BfpA in saline, induced a sustained and very effective antibody response. Anti-BfpA antibodies appeared in the blood after the first two antigen injections and were transferred to the egg yolks. The sustained anti-BfpA antibody titres, for long periods of time, allow the collection of a large number of eggs and, consequently, the availability of large quantities of antibodies. As the antibody avidity increases along with the immunization period and with the number of antigen injections [41, 42], the anti-BfpA antibodies obtained by this immunization schedule presumably interact strongly with BfpA. IgY antibodies were isolated from the egg yolks by a procedure combining precipitations in low-pH hypotonic water solution and 19% saturation of sodium sulfate. Further purification can also be obtained through chromatographic separations on Q-Sepharose FF. In all cases, the resulting concentrated IgY solution, as revealed by electrophoretic and Western blot analyses, exhibited the presence, in high titres, of anti-BfpA antibodies. Monospecific anti-BfpA antibodies were obtained by extensive immunoadsorption with *E. coli* BfpA-negative whole extracts. Such preparations were endowed with the ability to specifically recognize BfpA protein in a positive EPEC for the genes *bfpA* and *intimin* as EAF(+) but not in a negative EPEC strain for these genes as EAF(-) strain (Fig. 6). These observations were supported by the results from experiments performed using 12 stool specimens collected from children with diarrhoea as *E. coli* sources. Although positive for some of the EPEC O serogroups (Table 1) recognized by the World Health Organization [43], *E. coli* recovered from the children's stools were positive neither for those genes nor for the BfpA protein, as revealed by IgY anti-BfpA (Fig. 6). These IgY anti-BfpA preparations were also capable of inhibiting growth, *in vitro*, of *E. coli* expressing BfpA on their cell surface.

The monospecific IgY anti-BfpA antibodies prepared by the methods described herein are primarily directed for large-scale surveillance to detect the presence of EPEC in communities living in diarrhoea endemic areas.



## Acknowledgments

The authors thank Dr Regina Célia de Souza Campos Fernandes, 'Hospital Ferreira Machado', Campos dos Goytacazes, RJ, Brazil, the paediatrician engaged in the follow up of children with diarrhoea and Dr Nicola Conran for English review of the manuscript. This study was supported by FAPERJ, Project 'Cientista de Nosso Estado' Proc. number E-26/51.501/99. CMCA was the recipient of a FAPERJ scholarship, Proc. number E-26/150.830/97. AS was the recipient of a CAPES scholarship during the work developed at Centro de Pesquisa Gonçalo Moniz – Fundação Oswaldo Cruz, Salvador, Bahia, Brazil. MBN is a Senior Investigator of the Brazilian National Research Council (CNPq).

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