

Early acquisition of serum and saliva antibodies reactive to enteropathogenic *Escherichia coli* virulence-associated proteins by infants living in an endemic area

Carbonare CB, Carbonare SB, Carneiro-Sampaio MMS. Early acquisition of serum and saliva antibodies reactive to enteropathogenic *Escherichia coli* virulence-associated proteins by infants living in an endemic area.

Pediatr Allergy Immunol 2003; 14: 222–228. © 2003 Blackwell Munksgaard

Enteropathogenic *Escherichia coli* (EPEC) is the most common etiological agent of acute diarrhea among infants living in poor social conditions in Brazil and other developing countries. This infection is rare in breast-fed infants, as well as in children older than 2 years. Over the past few years, our group has attempted to identify antibodies to EPEC virulence proteins in human milk and to establish the *in vitro* protective role of these antibodies. In the present study, we report the identification of antibodies to EPEC virulence proteins in sera and saliva from children of different ages, living in slums in the city of São Paulo, Brazil. Using EPEC and bacterial constructs (pET) for immunoblotting (IB) analysis, antibodies reacting to the main adhesins (intimin, bundle-forming pili) and cell-signaling proteins (EPEC secreted proteins – Esp A, Esp B) were detected in sera from adults and children older than 1 year. Almost all children older than 1 year presented recognition patterns similar to those of adults in IB assays for serum IgG and secretory IgA antibodies, using EPEC outer membrane and other antigenic preparations. As previously observed for human milk, all samples from adults and older children recognized the 94 kDa molecular weight adhesin intimin strongly. In most children, previous EPEC symptomatic diarrhea could not be confirmed; however, almost all of them have presented one or more diarrhea episodes during their lifetime. These results suggest that reduction of EPEC infection frequency after 2 years of age may be associated with the development of anti-EPEC antibody repertoires.

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Key words: diarrhea; *Escherichia coli*; immunity; bacterial adhesins; antibodies

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Accepted 13 October 2002

Enteropathogenic *Escherichia coli* (EPEC) is the most common etiological agent of acute diarrhea among infants living in poor social conditions in Brazil and other developing countries (1, 2). Similarly to other intestinal infections, EPEC is rare among breast-fed infants (3, 4). In addition, this infection is rarely seen among children older than 1 year, and it is extremely rare in children older than 2 years, living in endemic areas (1, 4).

EPEC shows the characteristic pattern of localized adherence to epithelial cells *in vitro* (5).

The pathogenic mechanism of EPEC infection starts with bacterial adhesion to intestinal cells, followed by attachment and effacement lesion (A/E), which causes destruction of brush border microvilli and actin polymerization beneath the intimately adhered bacteria (2).

The bacterial structures responsible for the formation of the A/E lesion are encoded by chromosomal and plasmid loci. The chromosomal pathogenicity island LEE (locus of enterocyte effacement) contains several genes, including those associated with the type III secretion

system, the gene *tir*, which codes for the intimin receptor that is secreted by bacteria and translocated into the host cell, the *eae* gene, which codes for intimin, and the *esp* genes, encoding the secreted proteins EspA, EspB, EspC and EspD (2, 6, 7).

In the virulence plasmid EAF there is a cluster of genes associated with the fimbria BFP (bundle-forming pili). The gene *per*, positive regulator of expression of chromosomal genes encoding virulence factors, is also found in the EAF plasmid (2).

Over the past few years, our research group has focused on studying breast-feeding protective mechanisms against EPEC infection. We have shown that human milk presents a potent *in vitro* inhibitory activity on EPEC adhesion to HEp-2 cells. In addition, we have identified SIgA antibodies reactive to the main EPEC virulence-associated proteins as well as antilipopolysaccharide (LPS) antibodies in human milk (8–11). Antibodies reacting to EPEC adhesins (intimin alpha and beta, bundle-forming pili), to EPEC secreted proteins EspA and EspB, and to EPEC-encoded host cell intimin receptor (Tir – Translocated intimin receptor) were detected in colostrum and milk samples from mothers living in slums in São Paulo city (12–14). We have also demonstrated that colostrum IgA could act as a potent opsonin for EPEC killing by colostrum mononuclear phagocytes (15).

The mechanisms for the acquisition of anti-EPEC immunity are not fully understood, whether by means of experimental investigation or epidemiologic studies addressing individuals living in endemic areas and exposed to EPEC since early life. It has been recently demonstrated that serum samples from children 2 months to 3 years of age convalescing from EPEC diarrhea presented antibodies against intimin, EspA, EspB and BFP, whereas samples from healthy age-matched control children did not present these antibodies with equivalent reactivity (16).

In the present paper, we have investigated the presence of serum and saliva antibodies to the main EPEC virulence-associated proteins in infants and children 2 months to 2 years of age. Our results demonstrate that all children older than 1 year presented these antibodies, with reactivity patterns similar to those of adults living in the same area, and suggest that the early acquisition of antibodies to EPEC virulence factors may be associated with the reduced susceptibility to this infection observed after the first year of life.

Subjects, material and methods

Healthy infants

Serum and saliva samples from 70 infants 2 months to 2 years of age were kindly provided by Dr Olga A Takano. These samples were collected during a clinical trial carried out at the Hospital Israelita Albert Einstein, São Paulo, to test the efficacy of a *Hemophilus influenzae* b vaccine in a slum population, after parents' consent and approval by the Ethics committee of the hospital (17). All subjects were clinically healthy and well nourished. Sequential serum samples from some of these children were available. Previous EPEC symptomatic diarrhea could not be confirmed in most of these children; however, the majority of them had a history of one or more diarrhea episodes during their lifetime.

Normal adult serum pool and colostrum pool

Serum and colostrum pools were prepared with at least 20 samples collected from blood donors and healthy puerperal women between 48 and 72 h after delivery, respectively, at the University Hospital, USP. Most of these women also lived in a slum near the hospital.

Negative control

One serum sample from an 8-year-old patient with X-linked Agamaglobulinemia, prior to intravenous (i.v.) immunoglobulin treatment, served as a negative control.

EPEC strains and mutants

Bacterial strains used were wild-type EPEC 0111:H⁻, *eae*⁺, EAF⁺ e *bfp*⁺ isolated from an infant with acute EPEC diarrhea. BL21 – *E. coli* K12, transformed strains: BL21 – pET-intimin – super-expression of the gene *eae* (intimin), BL21 – pET-BFP – super-expression of BFP, BL21 – pET-EspA – super-expression of the gene *espA*, BL21 – pET-EspB – super-expression of the gene *espB*, kindly provided by Dr G Frankel (18). Mutant nonpathogenic strains, including *E. coli* obtained by transformation of EPEC O127: H6 (E2348/69), kindly provided by Dr M Donnenberg, were CVD 206 with *eae* deletion (19), CVD 452 with *sepB* deletion (20), UMD864 with *eaeB* deletion (21) presently called *EspB* (2) and JPN15 EAF plasmid cured strain (22).

Preparations of bacterial antigens were obtained from overnight cultures in brain–heart infusion (BHI) medium (Difco, Detroit, MI,

USA), centrifuged at 3000 g for 10 min. Bacteria were resuspended in phosphate-buffered saline (PBS) and the concentration adjusted to an $OD_{420} = 0.5$, corresponding to 10^9 bacteria/ml. This suspension was aliquoted in 1.3-ml microtubes and centrifuged at 13,000 g for 5 min. Pellets were resuspended in 150 μ l of sample buffer with 2% sodium dodecyl sulfate, 6 μ l of β -mercaptoethanol and 2 μ l of bromophenol blue, and the mixture was boiled for 10 min.

Bacteria containing pET constructs were grown overnight in 5 ml of Luria-Bertani (LB) medium with 30 μ g/ml kanamycin. Next, 10 ml of LB medium containing 0.2% glucose was subsequently inoculated with 100 μ l of the initial culture. After incubation for 2 h at 37°C, 24 mg of IPTG (isopropyl- β -D-thiogalactopyranoside) was added to the culture, and incubation at 37°C was maintained for 3–4 h, with shaking. Centrifugation and subsequent procedures were carried out as described above.

Western blotting assays for detection of IgG and IgA antibodies

Immunoblotting assays were performed as previously described (23). EPEC or transformed bacteria extracts were separated by SDS-PAGE in 10 or 12.5% polyacrylamide gels. Molecular-weight standards (Pharmacia, Uppsala, Sweden) were run in each gel. After transferring to nitrocellulose membranes for 40 min at 14 V in a semidry Trans-Blot SD apparatus (Bio-Rad, Hercules, CA, USA), strips were incubated overnight with 1 : 200 dilutions of serum or colostrum samples and 1 : 10 dilution of saliva

samples. Subsequently, strips were incubated with a 1 : 500 dilution of peroxidase-conjugated anti-human immunoglobulin antisera, for serum samples, or a 1 : 500 dilution of peroxidase-conjugated anti-human IgA for colostrum and saliva samples, for 2 h (Sigma, St Louis, MO, USA). Reactions were developed using the substrate in 3,3'-diaminobenzidine (Sigma).

Results

Adult serum pool contains antibodies that recognize the main EPEC virulence-associated proteins

Immunoblotting assays performed with a crude extract of EPEC wild-strain O111:H⁻ and mutant strains derived from O127:H6 lacking some virulence factors showed that adult normal serum pool contains IgG antibodies reactive to intimin, EspA, EspB, BFP and probably Tir. This reactivity was confirmed by immunoblotting assays carried out using bacterial constructs with super expression of the virulence factors intimin, EspA, EspB and BFP (Fig. 1).

Children present serum antibodies that recognize the main EPEC virulence-associated proteins

Immunoblotting (IB) analysis using wild EPEC extract and 46 serum samples from children 1 year and older showed that most of them presented a recognition pattern similar to that of the normal adult serum pool. The results are summarized in Table 1. Most sera showed strong recognition of the 94 kDa band. Few sera from infants younger than 1 year recognized all EPEC antigenic fractions. Analysis of sequential samples

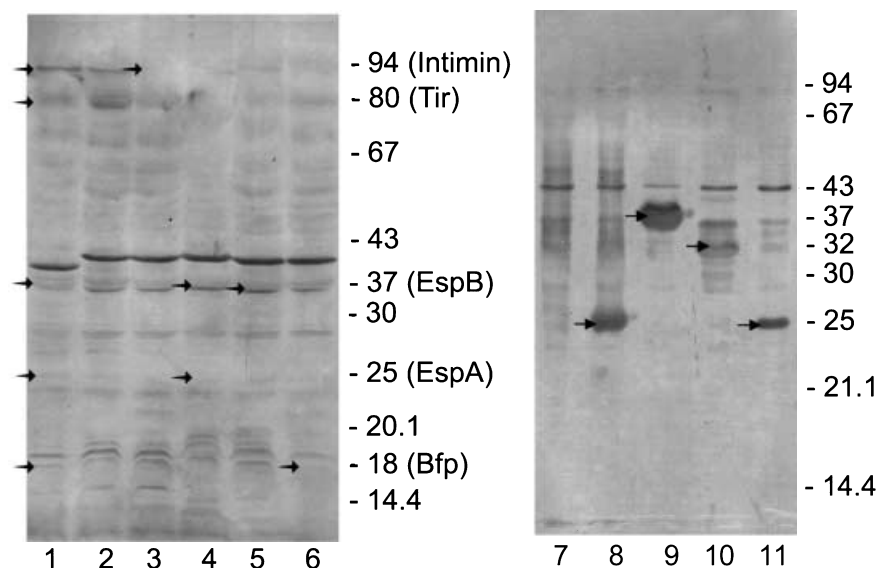


Fig. 1. Immunoblotting using SDS-PAGE 10–20% gradient gel. Antigenic preparations of EPEC and *E. coli* mutant strains were incubated with normal adult serum pool. (1) EPEC O111:H⁻, (2) E2348/69 O127:H6, (3) CVD206 (*eae*⁻), (4) CVD452 (*sepB*⁻), (5) UMD864 (*espB*⁻), (6) JPN15 (EAF⁻), (7) BL21, (8) BL21 pET-EspA, (9) BL21 pET-EspB, (10) BL21 pET-intimins, (11) BL21 pET-BFP.

Table 1. Number of serum samples with positive reaction in immunoblotting assay for EPEC antigenic fractions localized in molecular weight ranges (in rows)

MW (kDa)*	Age groups (months)					
	2-3 (n = 11)†	4-5 (n = 23)	7-9 (n = 25)	10-12 (n = 20)	13-18 (n = 27)	19-24 (n = 7)
94 (Intimin)‡	5	12	18	20	26	7
67-94 (Tir)‡	8	13	21	19	25	7
30-40 (EspB)‡	11	23	25	20	27	7
20-30 (EspA)‡	5	2	6	10	20	7
14-20 (Bfp)‡	7	9	15	17	26	7

*Molecular weight in kilodaltons.

†Number of children in each age group.

‡Region where is localized the virulence factor band.

Children are grouped in ages (columns).

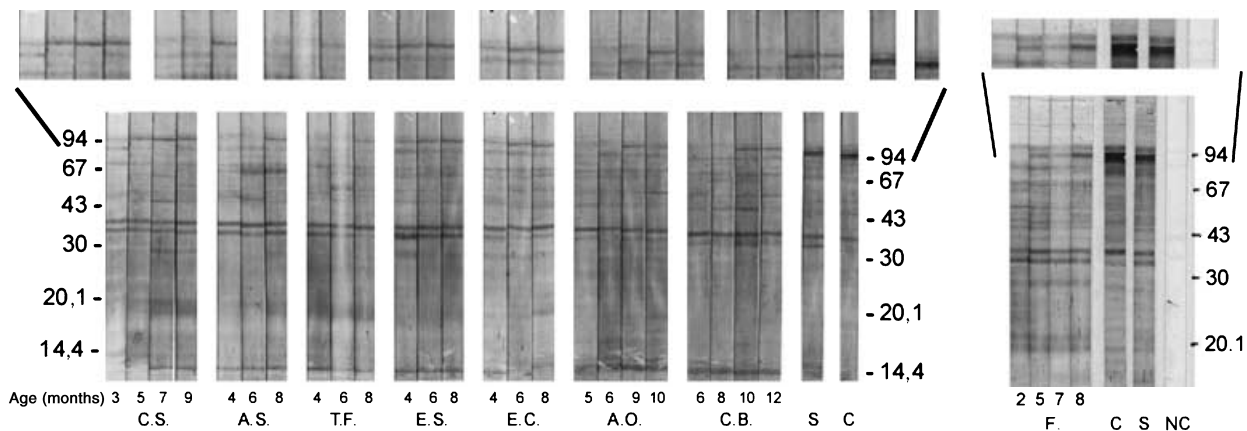


Fig. 2. Immunoblotting using 12.5% SDS-PAGE gel. Antigenic preparations of EPEC O111:H⁻ were incubated with serum samples from eight healthy children, (S) adult serum, (C) colostrum pools and (NC) negative control (serum sample from an agamaglobulinemia patient). Numbers under the strips correspond to the child's age in months, and the letters represent each child's initials. The strong 94-kDa band corresponds to intimin.

from the same children demonstrated acquisition of anti-intimin antibodies. In most cases, the intimin band is only present in later samples, giving a recognition pattern similar to that of the adult pool (Fig. 2 children CB, AO, AS and F). Recognition of bands corresponding to other EPEC virulence factors including EspA, EspB and BFP were also observed (data not shown).

Antibodies in serum and saliva of the same child have identical patterns of recognition of EPEC antigens

IgA antibody anti-EPEC virulence associated proteins can also be detected at an early age. IB analysis using saliva samples from children older than 1 year showed the presence of IgA secretory antibodies reactive to several EPEC antigenic fractions (Fig. 3). Comparative analysis of serum and saliva samples from the same child, collected at the same time, revealed similar reactivity patterns (Fig. 3).

Discussion

The aim of this study was to investigate the development of serum and secretory anti-EPEC antibodies in healthy Brazilian children, compared with the antibody repertoire of adults. First, we have shown the presence of antibodies reactive to EPEC virulence factors in a serum pool from healthy adults living in an area with a high frequency of EPEC infection. Antibodies reactive to the main adhesins (intimin, BFP) and cell-signaling proteins (EspA, EspB) were shown by IB assays using bacterial constructs (pET). We have also shown that serum and secretory antibodies reactive to EPEC virulence factors develop at an early age. Acquisition of an antibody repertoire equivalent to that of an adult occurred at around 12 months of age. At this age, a striking reduction in susceptibility to EPEC infection has been reported in endemic areas. The mechanisms for development of resistance to EPEC infection have not been well

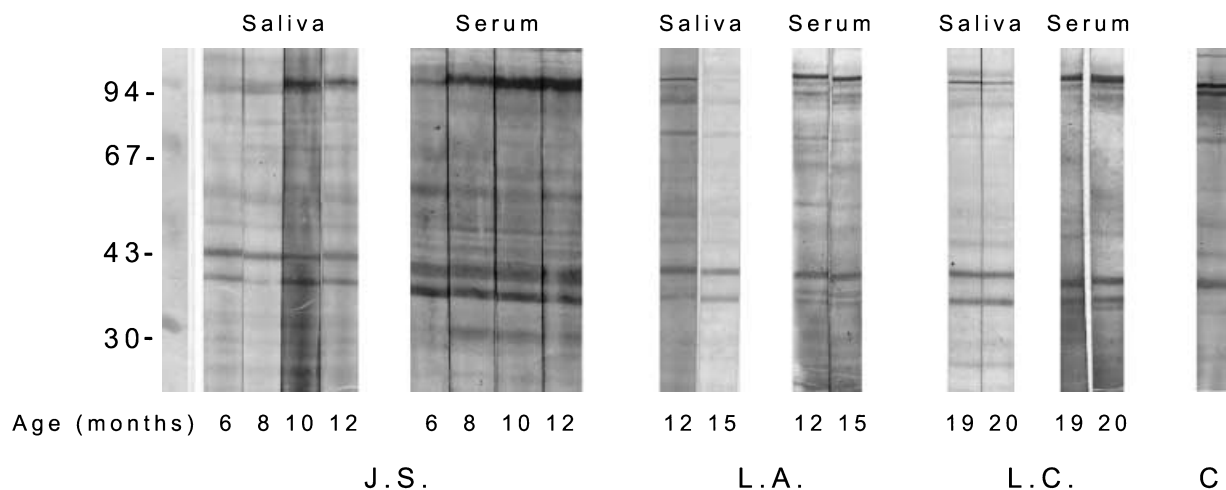


Fig. 3. Immunoblotting using 12.5% SDS-PAGE gel. Antigenic preparations of EPEC were incubated with paired serum and saliva samples from three healthy children and normal adult colostrum pool (C). Numbers under the strips correspond to the child's age in months, and the letters represent each child's initial. Saliva samples were assayed at 1 : 10 dilution. Colostrum and serum samples were assayed at 1 : 200 dilution. Peroxidase-conjugated antihuman IgA and anti-human immunoglobulins antisera were used at 1 : 500 and 1 : 750 dilutions, respectively.

characterized, and it has been hypothesized that resistance is due to acquired immunity, or alternatively to occurrence of constitutional changes in the host mucosa. Our findings reinforce the first hypothesis.

A plausible explanation for these findings is the intestinal colonization by the natural microbes, or the early contact with pathogenic bacteria even in the absence of symptomatic disease. Intestinal colonization with nonpathogenic *E. coli* represents efficient antigenic stimuli, leading to the production of secretory and serum antibodies (24). Besides EPEC, other enteropathogenic bacteria such as *Hafnia alvei*, *Citrobacter rodentium*, *Shiga toxin* producing *E. coli* (STEC) and other strains of *E. coli* have been isolated from human and animal intestines from rodents, pigs, dogs, lambs, rabbits and cows. These bacteria express intimin and are able to produce A/E lesions on epithelial cells (25).

IB assays with sequential samples from the same child showed that the earlier samples (24 months) did not recognize some antigenic fractions, as intimin and other EPEC virulence factors, whereas the later samples (8–12 months) revealed these bands. In most cases, samples collected about 12 months of age presented an antibody repertoire similar to that of the adult serum pool, with antibodies reactive to many EPEC antigenic fractions.

The recognition of a higher number of bands in some earlier samples (2–3 months) compared with later samples (4–6 months) suggests the presence of maternally acquired antibodies (Table 1).

Some bands are recognized by all samples in the molecular weight range of 30–40 kDa. This region includes EspB besides some porines very conserved between many bacterial strains (26). Antibodies reactive with these porines are found in all sera, saliva and colostrum samples.

A study also carried out in São Paulo City showed different data from ours. Martinez et al. (16) demonstrated that children convalescing from EPEC diarrhea presented serum antibodies reactive to virulence factors, whereas noninfected children of a similar age did not present these antibodies. In our population, it was not possible to determine whether children had previous EPEC infection. However, infections with EPEC are highly prevalent in communities living in poor hygiene conditions in the city of São Paulo, as well as in many other cities in developing countries. Due to the high frequency of low severity diarrhea episodes in this population, many children are not taken to health care services on these occasions. In addition, isolation of the etiologic agent is not always attempted, even in severe cases of diarrhea. It is possible that previous contacts with EPEC may have led to infections of different degrees of severity, including subclinical infections.

Mucosal IgA antibodies reactive to EPEC virulence factors *in vivo* inhibit bacterial adhesion and colonization by neutralizing the pathogenic effects of bacterial virulence associated factors. On the other hand, serum antibodies certainly do not play any significant role in the protection from EPEC diarrhea as the infection is limited to the mucosal surface of the digestive tract.

However, the serum anti-EPEC antibody repertoire mirrors the secretory antibody repertoire as shown by our findings comparing antibody specificities from serum and saliva samples collected simultaneously from the same children.

Antibodies to intimin may play a role in protection against EPEC disease, as reported by Levine et al. (27) in a study conducted with 10 volunteers experimentally infected. In one who did not become ill, the presence of anti-intimin antibodies in serum was shown before the challenge. The other nine volunteers who became ill had no pre-existing anti-intimin antibodies, but developed a strong reactivity to this adhesin after the challenge.

Other studies also carried out with adult volunteers have shown that the colonization with low virulence EPEC strains stimulated the production of serum anti-LPS antibodies (28). The fact that the second challenge with the same serotype resulted in disease with minor symptoms suggested that anti-LPS antibodies could be partially protective (29). The control serum samples collected from the volunteers before challenge did not show anti-EPEC antibodies, probably due to the low prevalence of EPEC infection in developed countries (27–29).

Our research group has focused on studying immunity against EPEC mediated by colostrum and milk. Breastfeeding is known to protect infants against many infectious diseases, including EPEC infection, so we have hypothesized that these secretions may contain elements of protective anti-EPEC immunity. We have previously demonstrated that human colostrum strongly inhibits *in vitro* EPEC adhesion to HEp-2 cells (8, 9) and that the inhibitory activity is associated with the IgA fraction (9, 10). Using techniques similar to those described in the present study, we have previously identified colostrum IgA antibodies reactive to the main EPEC virulence factors, including outer membrane proteins, fimbriae (10, 12–14) and LPS (11).

The strong recognition of a 94-kDa band, corresponding to the adhesin intimin, by all colostrum and milk samples, as well as all adult serum samples tested so far has been noteworthy. Our results provide evidence that IgA antibodies in colostrum reactive to intimin are able to inhibit *in vitro* EPEC adhesion to epithelial cells in culture (9, 10).

Currently, the most efficient prevention of EPEC infection is for the mother to continue with breastfeeding for as long as possible, or for at least 6 months, especially in the case of families of low socioeconomic level. After weaning, infants living in such conditions become

highly susceptible to EPEC infection, until immunity is acquired. The ultimate goal of our research group is to develop an effective anti-EPEC vaccine, which would benefit people exposed to infection particularly young children living in endemic areas.

Acknowledgments

We thank Professor Dr M L M Silva for EPEC strain and participation in the initial steps of this research, Dr O Takano for the children's serum and saliva samples, Dr G Frankel and Dr M Donnenberg for the bacterial strains. This work was supported by FAPESP.

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