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Antibody response against plasmid-encoded toxin (Pet) and the protein involved in intestinal colonization (Pic) in children with diarrhea produced by enteroaggregative *Escherichia coli*

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) is an emerging cause of pediatric and adult travellers diarrhea. The mechanism by which EAEC induce diarrhea is not completely known. Two serine protease autotransporter proteins, named Pet and Pic have been identified in EAEC strains. Pet has enterotoxic and cytotoxic activities, while the role of Pic in pathogenesis may lie on its mucinolytic activity. Little is known about Pet and Pic biological activities in vivo. In this study the antibody responses against these autotransporter proteins in convalescent children is investigated. Fifteen (83%) children showed specific antibodies against Pet or Pic in their sera. IgG and IgM antibodies were the main isotype found. Specific antibodies against Pic, but not against Pet, were detected in sera from age-matched control group. These data show that specific anti-Pet and anti-Pic antibodies are produced during the course of a natural EAEC infection in children.

Keywords: EAEC; Pet; Pic; Antibody response; IgG; IgM; IgA; Diarrhea; Children

1. Introduction

Enteroaggregative *Escherichia coli* (EAEC) is the emerging cause of pediatric and adult diarrhea, which has been associated with persistent enteric symptoms and also with travellers' diarrhea [1]. This enteric pathogen is characterized by its distinctive aggregative or "stacked-brick" pattern of adherence to cultured human epithelial cells (AA) and by the fact that it does not secrete the heat-label or heat-stable toxins of enterotoxigenic *E. coli* [2]. It is likely that this definition encompasses both pathogenic and nonpathogenic clones, which share factors conferring the common AA phenotype.

The heterogeneous pathogenicity of EAEC in humans has been confirmed in studies with volunteers [3], epidemiological studies of diarrhea [4–8] and outbreak investigations [9,10]. The EAEC factors which confer this heterogeneity have not been characterized, yet.

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The mechanism by which EAEC induce diarrhea is not well known. Most strains have cytotoxic activity on intestinal epithelium, characterized by dilatation of crypt openings, rounding of the enterocytes and exfoliation of mucosal epithelial cells when they are incubated with human colonic tissue [11]. These effects, combined with the secretory nature of EAEC-associated diarrhea, have led investigators to search for a cytotoxin and/or enterotoxin produced by EAEC strains.

A 108 kDa enterotoxin, termed plasmid-encoded toxin (Pet), was cloned and sequenced [12]. Pet is encoded by a large plasmid associated with AA expression in some EAEC strains and it is a member of the serine protease autotransporter class of proteins [2]. This protein was originally identified as an enterotoxin on rat jejunal tissue mounted in Ussing-chambers [13]. Another EAEC serine protease autotransporter, which was designated protein involved in intestinal colonization (Pic), has been identified in EAEC isolates [14]. The role of Pic in pathogenesis may be bound up with its mucinolytic activity. Moreover, the functional analysis of this factor also implicates it in serum resistance and hemagglutination [14], while Pet has enterotoxic and cytotoxic activities [15,16]. The products of the *pic* and *pet* genes are synthesized as 146.5- and 140-kDa precursor molecules which are processed at the N and C termini during their secretion, allowing the release of mature proteins (116- and 108-kDa, respectively) into the culture supernatants [12,14].

In spite of the important biological activities of these proteins, little is known about the in vivo production of Pet and Pic during a natural EAEC infection. Therefore, the aim of this study was to evaluate antibody responses against those proteins in children with diarrhea produced by EAEC.

2. Materials and methods

2.1. Patients and antisera

The study was approved by the local ethics committee, and informed consent was given by the study participants. We selected 18 children (ranging from 2 months to 3 years of age) with acute diarrhea, and with EAEC in feces as the only pathogenic agent. The children were attended in the University of São Paulo Hospital, in the city of São Paulo, Brazil [17]. The hospital provides free medical care to urban children of low socioeconomic status living in São Paulo. As controls it was studied seven children with diarrhea associated with different enteropathogen and 14 children without diarrhea at least a month. Patients and controls were attended in the same hospital, and were of the same age group and socioeconomic status. Blood was obtained from the children 10 days after the onset of acute diarrhea. Blood serum samples were kept at −20 °C until tested.

2.2. Bacterial strains and plasmids

The 18 *E. coli* strains isolated from 18 children selected for this study belong to several serotypes, but 10 strains had H18 antigen (Table 1). They were characterized as EAEC by adherence assay to HeLa cells [18] and by using the enteroagreggative adherence plasmid probe [19]. Pet and Pic expression by EAEC strains were sought in their culture supernatants by Western-blot assay by using specific rabbit anti-sera.

The following plasmids harbored in *E. coli* HB101 were employed: pCNFN1 [12] and pPic1 [14]. The antibiotics ampicillin (100 μg/ml) and tetracycline (10 μg/ml) were supplemented where appropriated. *E. coli* HB101

Table 1
Characteristics of diarrheagenic strains isolated from children and reactivity of their antibodies against Pet and Pic toxins

Isolates	Children's age	Serotypes	Pet expression	Anti-Pet antibodies	Pic expression	Anti-Pic antibodies
10	9m	O11:H18	–	IgM	–	IgM/IgG/IgA
12	1y 11m	O11:H18	+	IgM/IgG/IgA	–	IgM/IgG/IgA
27	10m	O125:H9	–	IgM/IgA^{w}	+	IgM/IgG/IgA
28	11m	O?:H18	–	IgM	+	IgG
29	9m	O15:H18	+	IgG^{w}	+	IgG
34	6m	O15:H18	+	IgA	+	IgG
35	9m	O15:H18	+	IgM/IgG/IgA	+	IgM/IgG/IgA
38	9m	O112:H?	–	IgM^{w}/IgG/IgA^{w}	–	IgG/IgA
47	3m	O77:H18	+	IgM	–	–
51	11m	O153:H2	–	IgM^{w}/IgG	+	IgG
57	2m	O86:H11	+	IgG^{w}	–	IgG
73	Unknown	O77:H18	–	IgM^{w}/IgG	–	IgM/IgG
92	5m	O?:H18	–	IgM^{w}/IgG^{w}	+	–
99	3m	O?:H18	–	IgM^{w}	–	IgG
131	2y 7m	O128:H35	–	–	–	–
145	7m	O92:H33	+	IgM	+	–
150	3m	O92:H33	+	–	+	IgM
164	6m	O106:H^{-}	+	–	+	–

y, years; m, months; ?, O or H antigens untypeable by conventional methods; w, weak reactions.

was used as negative control. All strains were kept at −70 °C in trypticase soy broth with 15% glycerol.

2.3. Serum antibodies against Pet or Pic

Nitrocelulose membranes containing either Pet or Pic proteins from HB101 (pCEFN1 or pPic1) supernatants were reacted with children's sera diluted 1:10 to determine the presence of Pet- or Pic-reacting antibodies as previously described [20]. Rabbit anti-Pet antiserum [13] or rabbit anti-Pic antiserum [14] were used as positive control. Serum samples from control children were also tested by the same methods and antigens. Goat anti-human IgG, IgM, IgA, and anti-rabbit IgG conjugated to horseradish peroxidase, were used as secondary antibodies.

3. Results

Among the 18 EAEC isolates, nine and ten strains expressed Pet and Pic in their culture supernatants, respectively, as detected by Western-blot using specific rabbit anti-Pet and anti-Pic antibodies (Table 1). Additionally, Table 1 shows the frequency of anti-Pet and anti-Pic antibodies according to the ages of the patients and the serotype of EAEC isolated.

3.1. Antibody responses to Pet

Fifteen sera (83%) from convalescent children contained anti-Pet antibodies, including seven from children infected with Pet-positive EAEC and eight from children infected with Pet-negative EAEC. Whereas, three sera did not contain antibodies against Pet, including two from children infected with Pet-positive EAEC (Table 1).

These specific antibodies belonged to different immunoglobulin isotypes. The isotype frequency of anti-Pet antibodies is shown in Table 2 and in Fig. 1(a). Interestingly, anti-Pet antibodies were found only in patients but not in control cases. Eight serum samples contained anti-Pet IgG antibodies and 12 serum samples contained IgM antibodies. These isotypes (IgM (67%) and IgG (44%)) were the main immunoglobulins found, while only five sera (28%) had IgA antibodies (Table 2). Among the nine children whose EAEC strains produced Pet, four had specific anti-Pet IgG and IgM antibodies in their sera (two sera contained both isotypes); whereas, two children sera had antibodies belonging to the three immunoglobulin isotypes (Table 1).

To confirm the identity of the 108 kDa protein recognized by the children sera, supernatant preparations from *E. coli* HB101 were probed with the samples, which had shown to contain anti-Pet antibodies. None of the 108 kDa protein-reacting sera recognized a 108 kDa protein in HB101 supernatant, confirming that the 108 kDa protein in HB101 (pCEFN1) recognized by the children sera corresponded to Pet.

3.2. Antibody responses to Pic

Thirteen sera (72%) from convalescent children contained anti-Pic antibodies, including seven from children infected with Pic-positive EAEC and six from children infected with Pic-negative EAEC. Whereas, five sera did not contain antibodies against Pic, including three from children infected with Pic-positive EAEC (Table 1).

As anti-Pet antibodies, specific anti-Pic antibodies belonged to different immunoglobulin isotypes. The isotype frequency of anti-Pic antibodies is shown in Table 2 and in Fig. 1(b). Unlike Pet, anti-Pic antibodies were found both in patients and in controls. In contrast with anti-Pet, anti-Pic IgM was less frequent than anti-Pic IgG antibodies. Twelve serum samples contained anti-Pic IgG antibodies (67%) while only 6 serum samples contained IgM antibodies (33%). Five sera (28%) had anti-Pic IgA antibodies (Table 2). Among the ten children whose EAEC strains produced Pic, six had specific anti-Pic IgG and three IgM antibodies in their sera (two sera contained both isotypes); whereas, three children sera (17%) had antibodies belonging to the three immunoglobulin isotypes (Table 1).

The serum samples employed as the control group were divided in two control subgroups. One of them were children with diarrhea associated with different enteropathogen other than EAEC (1–7) and the second were 14 children (8–21) without diarrhea at least a month. Anti-Pic antibodies were detected in 12 out of 14 samples from diarrhealess children (Fig. 2). Seven serum samples contained IgG, five IgM, and six IgA

Table 2
Anti-Pet and anti-Pic antibody isotypes in sera from patients and controls

Antibody isotypes	Anti-Pet				Anti-Pic			
	Patients (18)		Controls (21)		Patients (18)		Controls (21)	
	N	%	*N*	%	*N*	%	*N*	%
IgG	8	44	0	0	12	67	16	76
IgM	12	67	0	0	6	33	7	33
IgA	5	28	0	0	5	28	12	57

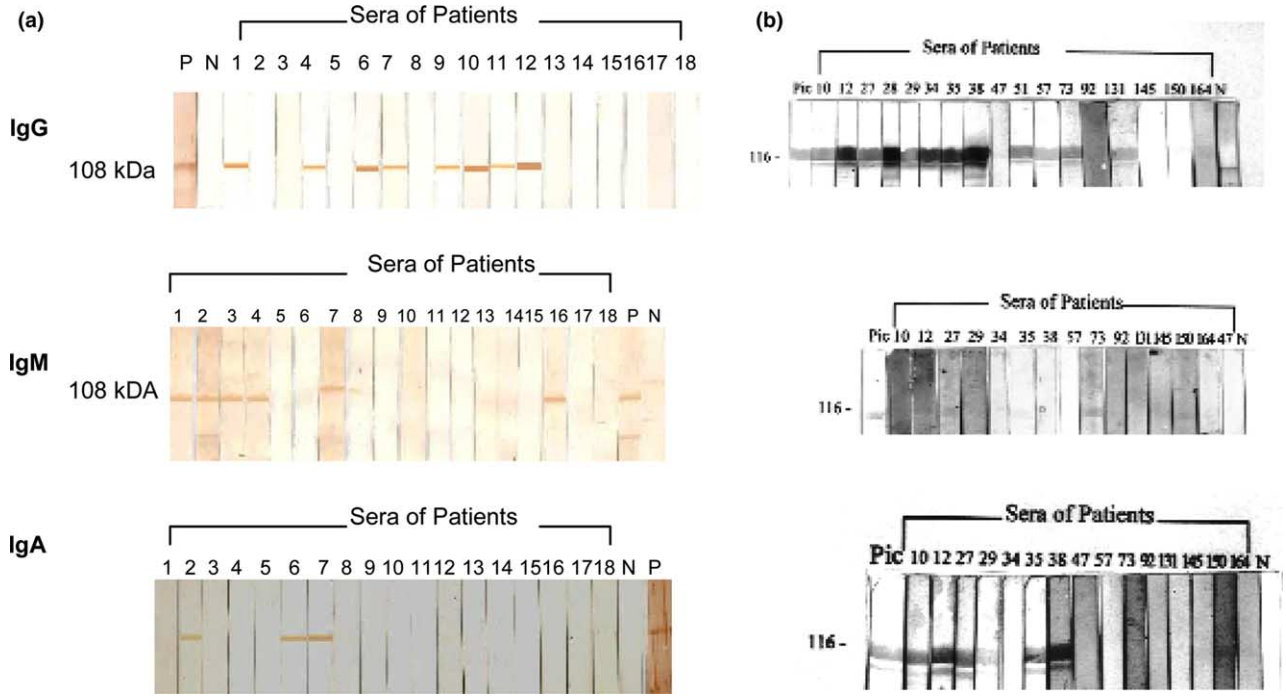

Fig. 1. Antibody response of children with EAEC infection against Pet (a) and Pic (b). (a) Preparation of HB101(pCEFN1) reacted with sera of children with diarrhea and anti-human antibodies. Positive control and N – negative control. (b) Pic preparation of HB101(pPic1) reacted with sera of children with diarrhea and anti-human IgG, IgM and IgA. Pic, positive control; N, negative control.

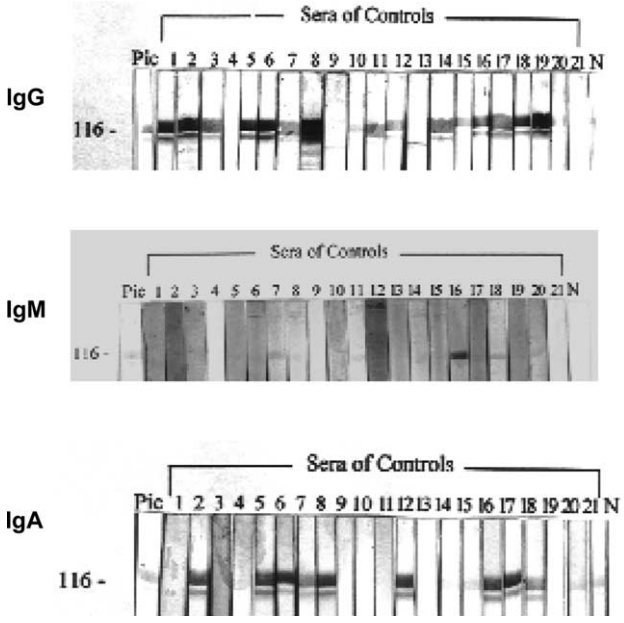

Fig. 2. Antibody response of children without EAEC infection against Pic. Pic preparation of HB101(pPic1) reacted with sera of diarrhealess children (lanes 8–21), and diarrheic children caused by other enteropathogens than EAEC (lanes 1–7) and anti-human antibodies. Pic, positive control; N, negative control.

antibodies. In seven serum samples of diarrheic children caused by other enteropathogens than EAEC, six had IgG antibodies against Pic, two IgM and six presented IgA.

Again, to confirm the identity of the 116 kDa protein recognized by serum samples, supernatant preparations from *E. coli* HB101 were probed with those samples that contained anti-Pic antibodies. None of the 116 kDa protein-reacting sera recognized the 116 kDa protein in *E. coli* HB101 supernatant, confirming that the 116 kDa protein in HB101(pPic1) recognized by the samples corresponded to Pic.

4. Discussion

Although the pathogenesis of EAEC infection is not completely known, several virulence markers have been identified in this category [1,21–23]. Among them, Pet and Pic may play important role in EAEC infections. The aim of the present study was to determine if specific antibodies against Pet and/or Pic are produced during the course of a natural EAEC infection in children. In order to address that, the reactivity of serum samples from convalescent children with diarrhea caused by EAEC were analyzed against these antigens, since the presence of antibodies against bacterial antigens has commonly been considered a marker of the production of virulence factors in vivo [20,24].

Our results show that both Pet and Pic induce an efficient antibody response, since only two and three strains out of 18 were unable to induce anti-Pet and anti-Pic antibody responses, respectively, in sera from children

containing Pet- and Pic-positive EAEC strains. These data suggest that both toxins may have a role in the pathogenesis of EAEC infections.

There was no correlation between the presence of Pet or Pic in the strains and the antibody response. This may be explained by preexisting infection with Pic- or Pet-producing EAEC. Studies performed by other authors have shown that the *pet* gene occurs in about 10–20% from the EAEC strains [1,6,22]. However, nine (50%) culture supernatants from our strains showed the presence of Pet and 83% of serum samples showed at least one specific antibody isotypes against Pet. Certainly, these anti-Pet antibodies became from exposition to strains producing Pet during the infection, since the control group did not show specific anti-Pet circulating antibodies. All these data suggest that Pet or *pet* gene has to be sought in fresh isolated EAEC, because *pet* is a plasmid-encoded gene and the plasmid may be lost. Interestingly, most of the isolated strains belong to a small number of clones, 56% of these strains have H18 antigen.

It is intriguing that control group did not show antibody response to Pet. These children lived in areas where EAEC is isolated in frequencies from 10 to 25% of the infant population [4–6,17,25]. Furthermore, it is possible that anti-Pet antibodies do not remain in circulation for long periods of time as it has been reported for anti-BFP antibodies [20]. It is also important to note that Pet is a protein encoded in the EAEC plasmid while Pic is chromosomally encoded.

It is interesting to observe that the main circulating anti-Pet antibody isotype is IgM. Normally, in a humoral response to an infection, this isotype is the most precociously produced antibody but on the other hand it is the first one to disappear [26]. The sera of these children were obtained in the tenth day of diarrhea, which would explain the presence of these antibodies. Follow-up studies for at least six months should be carried out in order to determine the antibody production curve in the humoral response to intestinal infection by EAEC.

The high frequency of anti-Pic antibodies was somewhat surprising. Contrary to Pet, specific antibodies against Pic were detected in sera from healthy children without diarrhea. Considering that all children live in an area in which EAEC is endemic and therefore would be likely exposed to EAEC infection, the anti-Pic antibodies may remain circulating for a long period of time unlike Pet antibodies. Another possibility would be the production of Pic by other bacteria from the normal flora or from other origins, such as *Shigella* or uropathogenic *E. coli*, which produce a Pic homolog protein [14,27,28]. It is also possible that Pic cross-react with other bacterial antigens.

The data presented here strongly suggest that specific antibodies are elicited during naturally occurring infections with EAEC among young children and that Pet and Pic are produced in vivo by different EAEC serotypes. These are important findings since only one study has shown antibodies against Pet in two patients belonging to the same Mexican diarrhea outbreak [13].

Pic induces an efficient antibody response, since anti-Pic antibodies may remain circulating for long periods. Sera from children without diarrhea showed specific IgA and IgM antibodies against Pic. IgA antibodies prevent the attachment of bacteria or toxins to epithelial cells, and provide the first specific defense against a wide variety of pathogens. Preexisting high intestinal sIgA titers were associated with protection from acquiring EAEC-induced diarrhea [7]. Furthermore, Gomez et al. [29] showed that high intestinal sIgA titers against an EAEC strain were associated with protection from symptomatic infection. The development of protective antibodies during the course of an infection remains an interesting issue to address. Studies are needed to elucidate whether these antibodies are protective.

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