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Surveys of human enterotoxigenic *Escherichia coli* from three different geographical areas for possible colonization factors

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SUMMARY

Enterotoxigenic *Escherichia coli* (ETEC) from Burma, central Africa (Rwanda and Zaire) and Peru, were screened by enzyme-linked immunoassays for the colonization factor antigens (CFAs) and putative colonization factors (PCFs): CFA/I, CFA/II, which consists of three coli surface-associated (CS) antigens, CS1, CS2 and CS3, CFA/III, CFA/IV (CS4, CS5, CS6), CS7, PCFO9, PCFO159.H4, PCFO166, and CS17. The highest proportion of ETEC with identifiable colonization factors (71%) were found in the strains from Burma, which were mainly positive for CFA/I (38%), but strains producing CFA/II (4%), CFA/IV (11%), CS7 (10%), CS17 (4%), PCFO159.H4 (2%) and PCFO166 (2%) were also found. Sixty-nine percent of the ETEC from central Africa were positive for known colonization factors. While CFA/I positive strains were important (12%), a higher number of ETEC producing CFA/IV (33%) and CS17 (24%) were found. Fifty-two percent of the Peruvian strains produced identifiable colonization factors. The largest group of strains produced antigens of the CFA/IV complex (17%), while ETEC producing CFA/II (6%), CFA/III and CS6 (2%), CS7 (6%), PCFO9 (6%), PCFO166 (8%) and CS17 (7%) were also found. These surveys show that there is a considerable variation in the proportions and types of colonization factor found in different geographical areas. From 29 to 48% of the ETEC did not possess an identifiable colonization factor. These were particularly of the LT only producing type. These results have important implications for vaccine formulation.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoeal diseases in infants in developing countries and in visitors to those countries. Their pathogenicity is due to their ability to adhere to the intestinal mucosa of the duodenum and to produce enterotoxins. The factors which mediate adherence to the intestinal epithelial cells are called colonization factors and they are usually fimbriae many of which have the ability to agglutinate red blood cells from a number of animal species in the presence of mannose [1]. The first colonization

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factor, termed colonization factor antigen I (CFA/I) was identified in 1975 as a single fimbrial antigen [2] and was found on *E. coli* which produce heat-stable (ST) or ST and heat-labile (LT) enterotoxins. Since that time other factors have been identified. Two groups of antigens have been associated with ST^+ or ST^+ LT^+ strains. Strains producing CFA/II possess one or other of the fimbrial coli surface-associated (CS) antigens CS1 or CS2 and a fibrillar antigen CS3, or may possess CS3 alone [3, 4]. ETEC producing CFA/IV may have the fimbrial antigen CS4 or CS5 and a second antigen, CS6 [5] or they may produce CS6 without CS4 or CS5 [6]. A number of fimbriae have been described which have similar properties to the well-characterized antigens CFA/I, CFA/II and CFA/IV. These fimbriae are potentially colonization factors although they have not been tested for their ability to colonize man. These factors, which are antigenically distinct comprise: CFA/III found on LT^+ strains [7], putative colonization factor (PCF)O159.H4 (PCFO159) on ST^+ LT^+ *E. coli* [8], PCFO166 on ST^+ and ST^+ LT^+ strains [9], CS17 on LT^+ strains [10], CS7 found on *E. coli* 334 [11, 12] and other ST^+ LT^+ and LT^+ strains and PCFO9 found on LT^+ *E. coli* [13].

Surveys of ETEC have shown that some colonization factors, i.e. CS1 or CS2 with CS3, CS4 with CS6, CFA/III with CS6 and PCFO159, are associated with one or two serogroups of *E. coli* [3–5, 14, 15]. The range of serogroups associated with other colonization factors, i.e. CFA/I, CS3 as a single antigen, CS5 with CS6, CS6 without CS4 or CS5, CS7, PCFO166 and CS17 is much more diverse, ranging from about 5 to 15 [6, 9, 10, 12, 14, 16, 17].

Experiments in rabbits and humans have shown that CFA/I and the CS antigens of CFA/II and CFA/IV are protective antigens which have been considered as potentially useful for vaccines [18–20] and it is likely that other colonization factors could have a similar function. It is therefore important to know the proportion of ETEC in a particular area which possess colonization factors and the type of factor they produce. Early surveys of ETEC for the presence of CFA/I gave varying results. An initial study found that 86% of ETEC isolated from students becoming ill when visiting Mexico produced CFA/I [21] but when a collection of ETEC from several countries and of different serotypes were tested a much lower proportion of strains were found to produce this antigen (22). Surveys of ETEC for the production of CFA/I, CFA/II and in some cases CFA/IV again gave varying results, from 79% colonization factor positive in a small study in Chile [23], 75% in Bangladesh [16], 29% in Thailand [14, 17] to 23% in travellers returning to Japan from South-East Asia [24].

Variations in the proportions of the colonization factor positive ETEC found has been ascribed to loss of plasmids coding for CFAs during storage or subculture [14] but may also be due to the fact that the strains were not screened for the new colonization factors or that they did not possess any known factor. We have now examined batches of strains from three different geographical areas, Burma, central Africa (Rwanda and Zaire) and Peru to determine the prevalence of all the colonization factors described above. All the cultures were checked for the presence of toxin genes at the time of screening.

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MATERIALS AND METHODS

Bacterial strains

The ETEC to be screened for colonization factors were isolated from diarrhoeal cases in Burma, central Africa (Rwanda and Zaire) and Peru. They were checked for enterotoxin production and serotyped in the Division of Enteric Pathogens.

O and H serotyping

The ETEC were tested with antisera for O groups 1–170 and for 53 H antigens [25].

Enterotoxin testing for ST and LT

Two methods of testing were used for each enterotoxin. Initially ST production was detected in the infant mouse [26] and LT production with Y-1 adrenal cells in tissue culture as previously described (27). Later DNA probes were also used to check that the strains still carried toxin genes. These probes comprised synthetic oligonucleotides labelled with alkaline phosphatase and supplied in kit form (SNAP^r system; DuPont-NEN). The ST probe contained both STA1- and STA2-specific sequences. Hybridization to colonies was done by methods recommended by the manufacturer [28].

Preparation of antisera

Standard ETEC strains which were positive for CFA/I, CS1–CS3, CFA/III, CS4–CS6, CS7, PCFO9, PCFO159, PCFO166 and CS17 were used to prepare a formalinized suspension containing about 10^9 bacteria ml^{-1} for use as vaccines in rabbits as previously described [4]. Specific antisera were prepared by absorbing them with the appropriate colonization factor-negative variants.

Enzyme-linked immunosorbent assays (ELISA)

Strains to be tested in ELISAs were streaked on CFA agar slopes containing bile salts (1·5 g/l) [9]. The cultures were suspended in 2 ml 0·05 M carbonate/bicarbonate buffer (pH 9·6) and heated at 60 °C for 30 min. The ELISAs were done as previously described [15]. The heated suspensions were used to coat microtitre plates. Colonization factors were identified with specific absorbed antisera or IgG. Binding of the antibody was detected using anti-rabbit IgG peroxidase conjugate. The substrate was prepared by adding 9 mg *o*-phenylenediamine and 40 μl 30% (v/v) H_2O_2 to 25 ml 0·1 M sodium phosphate buffer (pH 6·0); 100 μl was added to each well and the plates were incubated in the dark at room temperature for 10 min. Sulphuric acid (2 M, 25 μl) was added to each well to stop the reaction. Initially the strains were tested for CFA/I, CS3, CFA/III, CS6, CS7, PCFO9, PCFO159, PCFO166 and CS17. They were not screened for CS1, CS2, CS4 or CS5 because apart from a few CS2-positive strains described by Smyth [3] all CS1 and CS2 producing strains also had CS3 [3, 4] and all CS4- and CS5-producing strains had CS6 [5, 14]. After the initial screening CS3- and CS6-positive strains were tested with antisera against CS1 and CS2 or CS4 and CS5 as appropriate.

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Table 1. *Colonization factors of ETEC isolated in Burma*

Colonization factor	Serotype	Enterotoxin	No. of strains*
CFA/I	O126.H12	ST	3 (8)
	O128.H12	ST	12 (30)
CS2, CS3	O6.H16	ST, LT	1 (2)
CS3	O8.H9	ST, LT	1 (2)
CS4, CS6	O25.H42	ST, LT	2 (5)
CS5, CS6	O115.H5	ST	1 (2)
CS6	O27.H$^-$	ST	1 (2)
	O169.H$^-$	ST	1 (2)
CS7	O114.H$^-$	LT	3 (8)
	O114.H rough	LT	1 (2)
PCFO159	O159.H20	ST, LT	1 (2)
PCFO166	O20.H$^-$	ST, LT	1 (2)
CS17	O8.H9	LT	1 (2)
	O167.H5	LT	1 (2)
No. of strains with colonization factors			30 (71)
Total no. of strains tested			42

Twelve strains did not possess colonization factors: LT$^+$, O7.H18 (2); O17.H51 (1); O128.H12 (2); O?.H7 (1); ST$^+$, O8.H$^-$ (1); O126.H30 (1); ST$^+$ LT$^+$, O6.H16 (1); O8.H21 (1); O17.H18 (2).

* Numbers in parentheses are percentages of strains possessing colonization factors.

Table 2. *Colonization factors of ETEC isolated in central Africa (Rwanda and Zaire)*

Colonization factor	Serotype	Enterotoxin	No. of strains*
CFA/I	O127.H21	ST	13 (12)
CS5, CS6	O29.H21	ST	9 (8)
CS6	O9a.H21	ST	11 (10)
	O27.H7	ST	7 (7)
	O30.H10	ST	7 (7)
	O104.H rough	LT	1 (1)
CS17	O8.H9	LT	17 (15)
	O114.H21	LT	10 (9)
No. of strains with colonization factors			75 (69)
Total no. of strains tested			107

Thirty-two strains did not possess colonization factors: LT$^+$, O8.H9 (9); O64.H$^-$ (5); O88.H25 (3); O105.H18 (6); O136.H9 (1); O141.H27 (2); O141.H$^-$ (1); ST$^+$, O76.H21 (5).

* Numbers in parentheses are percentages of strains possessing colonization factors.

RESULTS

Forty-two strains from Burma were examined; they included 20 ST$^+$, 12 LT$^+$ and 10 ST$^+$ LT$^+$, *E. coli*. (Table 1). Thirty-eight percent of the total *E. coli* tested produced CFA/I and 80% of these belonged to serotype O128.H12. These ST$^+$ CFA/I$^+$ strains were the most common type. The other most frequent groups of strains were those producing CFA/IV (11%), CS7 (10%), and CFA/II (4%). Twelve strains (29%) were negative for all the colonization factors. Six of these strains were LT$^+$, two were ST$^+$ and four were ST$^+$ LT$^+$.

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Table 3. *Colonization factors of ETEC isolated in Peru*

Colonization factor	Serotype	Enterotoxin	No. of strains*
CS2, CS3	O6.H16	ST, LT	2 (4)
CS3	O8.H$^-$	ST, LT	1 (2)
CFA/III, CS6	O25.H$^-$	LT	1 (2)
CS4, CS6	O25.H$^-$	ST, LT	1 (2)
CS5, CS6	O128.H2	ST	2 (4)
CS6	O128.H$^-$	ST	3 (7)
	O148.H28	ST, LT	1 (2)
	O?.H1	ST	1 (2)
CS7	O103.H49	LT	1 (2)
	O114.H49	LT	2 (4)
PCFO9	O9.H$^-$	LT	1 (2)
	O143.H19	LT	1 (2)
	O?.H$^-$	LT	1 (2)
PCFO166	O78.H18	ST	2 (4)
	O166.H27	ST	2 (4)
CS17	O8.H9	LT	3 (7)
No. of strains with colonization factors			25 (52)
Total no. of strains tested			47

Twenty-two strains did not possess colonization factors: LT$^+$, O3.H1 (1); O3.H9 (1); O8.H$^-$ (2); O41.H30 (1); O80.H10 (1); O159.H4 (2); O?.H7 (1); O?.H19 (3); O?.H26 (1); O?.H$^-$ (1); ST$^+$, O8.H1 (1); O?.H$^-$ (1); ST$^+$ LT$^+$; O109.H45 (4), O113.H$^-$ (1); O?H$^-$ (1).

* Numbers in parentheses are percentages of strains possessing colonization factors.

Fifty-two ST$^+$ and 55 LT$^+$ *E. coli* were received from central Africa (Table 2). Among the *E. coli* on which a colonization factor was detected were ST$^+$ strains producing CS antigens of the CFA/IV complex (33%), LT$^+$ CS17$^+$ *E. coli* (24%) and ST$^+$ CFA/I producing *E. coli* of serotype O127.H21 (12%). One LT$^+$ *E. coli* of serotype O104.H round produced CS6. No other colonization factors were detected. Of the 32 strains (31%) which did not produce a known colonization factor, 27 were LT$^+$ of 7 different serotypes and 5 were ST$^+$ of serotype O76.H21.

The Peruvian *E. coli* consisted of 12 ST$^+$, 24 LT$^+$ and 11 ST$^+$ LT$^+$ strains. CFA/I producing strains were not detected in the group tested here (Table 3). The largest group of strains (17%) were CFA/IV positive. The other PCF producing strains were of seven different types. Among the 22 strains (48%) which did not produce known colonization factors, 14 were LT$^+$ of 10 different serotypes, 2 were ST$^+$ of 2 serotypes and 6 were ST$^+$ LT$^+$ of 3 serotypes.

DISCUSSION

The highest proportion of ETEC with colonization factors were detected in the Burmese strains. As in previous surveys of Asian strains [14, 16, 17] CFA/I$^+$ strains formed the largest group (38%). Testing for new colonization factors CFA/III, PCFO159, PCFO166, CS7, CS17 and PCFO9 increased the number of identifiable colonization factor positive strains by 17%. Among the central African ETEC CFA/I$^+$ strains also formed a substantial proportion (12%), however the largest group of ETEC with an adhesion factor possessed CS antigens

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of the CFA/IV complex (33%) and the new factor, CS17 (24%). There were no CFA/II-producing strains, which correlated with the fact that no ST^+ LT^+ strains were received. Previous work has shown that the plasmids which code for CFA/II also code for ST and LT [29]. The Peruvian ETEC were very mixed; no CFA/I^+ strains were detected; the largest group (15%) of CFA positive ETEC possessed antigens of the CFA/IV complex.

In the three surveys detection rates for possible colonization factors of 52, 69 and 71% were achieved. Several reasons can be advanced to explain why these detection rates were not higher. Most plasmids which code for CFAs also code for toxin production [8, 9, 28–31). We reduced the loss of CFAs due to plasmid instability by testing for toxigenicity immediately prior to ELISA testing and by only using positive toxin producers. However alterations can also occur to plasmids that result in loss of CFA without loss of toxin production [28] so that strains which were originally CFA and toxin producing may now only be toxigenic. Such an alteration probably occurred in an ST^+ LT^+ strain of serotype O6.H16 from Burma.

It has also been suggested that some strains which produce only LT may not be pathogenic and may never have possessed a colonization factor since in some surveys they are found as frequently in controls as patients with diarrhoea and because they are found in a wide variety of serotypes [17, 32, 33]. In these surveys also the strains in the greatest variety of serotypes are LT only producers and some of these strains may be non-pathogenic. However among the strains we have screened were several LT^+ strains with the putative colonization factors, CFA/III^+ CS6, PCFO9, CS7 and CS17. An LT^+ strain of serotype O25.H^- like the CFA/III^+ strain has been shown to be pathogenic [34] and experiments using the RITARD model suggest that strains producing two of these factors, CS7 and CS17 are able to colonize rabbits (A.-M. Svennerholm, personal communication). Much of the original work on colonization factors was done on strains which came from Asia. Some of the strains from Africa and South America which apparently do not possess colonization factors are of serotypes uncommon in these Asian strains and they may well possess new colonization factors.

Although the number of strains tested was small, the surveys show the great diversity of serotypes of ETEC in the different geographical areas. The variation in the types of colonization factors found between regions is much less. Relatively fewer colonization factors than O groups would be required to cover the same number of ETEC; an important consideration for the construction of a vaccine. These and other surveys have shown that the majority of the ETEC in each area which do not have detectable colonization factors produce LT. In a large field trial of an oral vaccine cholera-B subunit was shown to be protective against ETEC producing LT [35]. A prototype oral vaccine consisting of a mixture expressing the most important CFAs and cholera-B subunit is currently being tested in volunteers in Sweden (A.-M. Svennerholm, personal communication).

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