COLONISATION FACTORS AMONGST CLINICAL ISOLATES OF ENTEROTOXIGENIC *ESCHERICHIA COLI*

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ABSTRACT

The production of heat-labile (LT) and heat-stable (ST) enterotoxins, colonisation factor antigens (CFAs) and haemagglutinins was investigated amongst 310 Escherichia coli (E. coli) isolates obtained from 62 children under the age of five, with diarrhoea. Twenty-one isolates were found to produce enterotoxins, of which fifteen (71%) isolates produced ST only, 2 (10%) produced LT only and 4 (19%) produced both LT and ST. However, none of the isolates demonstrated any of the common CFAs identified to date, but 8 out of the 21 isolates demonstrated haemagglutination with rabbit, sheep or human group A erythrocytes, suggesting the presence of putative CFAs, yet unidentified.

Keywords: *colonisation factor antigens, enterotoxins, Escherichia coli, haemagglutinins, diarrhoeal disease.*

SINGAPORE MED J 1995; Vol 36: 495-497

INTRODUCTION

Diarrhoea caused by enterotoxigenic *Escherichia coli* (ETEC) is an important health problem, particularly in infants and children under the age of five in developing countries and in travellers to these areas[1]. ETEC produce enterotoxins, which comprise a high molecular weight, immunogenic, heat-labile toxin (LT) and a low molecular weight, non-immunogenic, heat-stable toxin (ST), which may occur either singly or in combination amongst strains[2]. The LT and ST of ETEC have been well defined biochemically and genetically[3].

In addition to enterotoxins, ETEC also produce colonisation factor antigens (CFAs), which are usually proteinaceous fimbriae present on the bacterial cell surface, necessary for adherence of the bacterium to enterocytes[1,3]. Of the CFAs identified to date only CFA I, CFA II and CFA IV have been well characterised[4]. CFAs are often encoded on high molecular weight plasmids and each CFA is usually associated with a particular toxotype[5,6]. For example, genes encoding CFA I expression have been associated with plasmids coding for ST while genes encoding CFA II expression have been associated with plasmids coding for both LT and ST[7].

The role of CFAs in protective immunity has also been widely described, suggesting the importance of these antigens in the development of vaccines against ETEC[4,8].

The aim of this study was to investigate the occurrence of ETEC, the prevalence of toxotypes and the distribution of CFAs or putative colonisation factors (PCFs) amongst ETEC in diarrhoeal cases in children under five years of age, at the University Hospital, Malaysia.

METHODS

Bacterial Strains

A total of 310 isolates from 62 cases of diarrhoea in children under five years of age were studied over a period of six months at the University Hospital, Kuala Lumpur. Briefly, stool specimens were cultured on MacConkey agar plates for selection of *E. coli*. Ten lactose fermenting colonies, presumptive of *E. coli* were subcultured onto Blood agar (BA) plates and incubated for a further 18-24 hours at 37°C. From each BA plate five colonies were selected and tested directly by an enzyme linked immunosorbent assay (ELISA), for LT and ST production[9,10]. Isolates positive for enterotoxin production were maintained on nutrient agar (NA) slopes at 4°C and at -70°C in nutrient broth (NB) supplemented with 50% glycerol until analysed for the presence of CFAs[3,11,12]. The following *E. coli* strains were included as controls: 325542 (ST^+; CFA I), 60R936 (LT^- / ST^-; CS 1), 58R957 (LT^- / ST^-; CS 2), E19446 (LT^+ / ST^+; CS 3), E170181A (ST^+; CS 5), 62R486 (ST^+; CS 4), E34420C (LT^- / ST^-; CFA III), 350C1A (LT^+ / ST^+; PCF 0159), E7476A (ST^+; PCF 0166), E29101A (LT^+; CS 7), AND E20738A (LT^+; CS 17) [University of Goteborg, Sweden].

LT and ST Detection

Detection of LT and ST was performed using GM1 - ELISA methods as described previously[9,10]. The LT assay is a direct ELISA whereas the ST assay is an inhibition ELISA. Briefly, five individual colonies of each strain, from BA plates were inoculated into individual wells of 96-well microtitre plates [precoated with GM1 and blocked with 0.1% bovine serum albumin (BSA)] containing Luria Bertani (LB) medium (100 µl/well with lincomycin (45 µg/ml) and glucose (2.5 mg/ml). The plates were incubated with shaking at 37°C, overnight. The culture medium (50 µl) was then transferred to corresponding well on new GM1-plates, coated with ST-cholera toxin B subunit conjugate (ST-CTB; 1:300), for the detection of ST[10]. The original plate used for culture was then assayed for GM1 - bound LT, by the addition of monoclonal antibody (MAb) against LT (University of Goteborg, Sweden), followed by goat anti-mouse Ig-HRP conjugate (Bio-Rad, USA) and enzyme substrate (Orthophenyldiamine). From the LT assay, colonies giving absorbance values greater than or equivalent to 0.1 above background values, were considered LT positive.

The presence of ST was determined in the ST-CTB coated plate by the GM1 - ELISA inhibition assay as described previously[10], using a MAb against STa (University of Goteborg, Sweden). From the ST assay, ST positive colonies were identified as those giving absorbance values equivalent to or less than 50% of negative control values.

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Detection of CFAs

Toxigenic *E. coli* isolates (as determined by the toxin assays previously) were assayed for the expression of CFAs, using the method described by Evans et al in 1975[13]. Briefly, each toxigenic isolate (stored at -70°C) was streaked onto CFA agar with or without bile salts[13] and incubated overnight at 37°C. Subsequently, a loopful of each culture was suspended in 10 µl of saline on a glass slide to which 10 µl of specific anti-CFA MAb was added. The slide was tilted back and forth for mixing and the appearance of macroscopically visible agglutination within two to three minutes was regarded as a positive reaction. Bacterial suspensions in saline without the addition of MAbs were included as negative controls in order to exclude autologous agglutination. The specific anti-CFA MAbs used included anti-CFA I, anti-CFA III, anti-CS 1, anti-CS 2, anti-CS 3 and anti-CS 4 for isolates grown on CFA agar without bile salts; anti-CS 5, anti-PCF 0159, anti-PCF 0166, anti-CS 7 and anti-CS 17 for isolates grown on CFA agar with bile salts [University of Goteborg, Sweden].

Haemagglutination tests

All ETEC isolates negative in the CFA analyses were tested for the presence of haemagglutinins[11,12] using fresh rabbit, sheep and human group A erythrocytes. Each colony was suspended in 10 µl of saline on a glass slide to which 10 µl of 3% (v/v) erythrocyte suspension in saline with or without 1% D-mannose, was added. The slide was tilted back and forth for mixing and observed for haemagglutination (HA) for two to five minutes. Results were recorded as (+) or (-). The HA was considered mannose-sensitive (MSHA) if HA was observed only in the erythrocyte suspension without 1% D-mannose. However, the HA was considered mannose-resistant (MRHA) if the same degree of HA occurred in the presence and absence of 1% D-mannose[14].

RESULTS

A total of 310 *E. coli* isolates from 62 diarrhoeal cases in children under the age of five, were analysed for toxin production. Of these, 21 (6.8%) isolates, obtained from 11 (18%) of the cases were found to be toxigenic. Fifteen (71%) isolates produced ST only, 2 (10%) produced LT only and 4 (19%) produced both LT and ST (Table I). In relation to the 11 cases, it was found that ST-only producing ETEC was isolated in 7 (63.6%) cases whereas both ST-ETEC and LT/ST-ETEC were isolated in the 4 (36.4%) other cases. The remaining 289 *E. coli* isolates did not produce either toxin. All control strains used were stable and gave reproducible results over the period of 6 months.

The 21 ETEC isolates were analysed for the presence of CFA I, CFA III, CS 1, CS 2, CS 3, CS 4, CS 5, PCF 0159, PCF 0166, CS 7 and CS 17 in a slide agglutination assay using specific anti-CFA MAbs. It was observed that none of the isolates demonstrated any of the common or less frequently occurring CFAs tested for.

However, when these 21 CFA-negative ETEC isolates were tested for the presence of haemagglutinins, using rabbit, sheep and human group A erythrocytes, differing HA patterns were observed in 8 (38%) of them. The remaining 13 (62%) isolates (2 LT producers and 11 ST producers) did not agglutinate any of the erythrocytes tested. It was also observed that the ST producing ETEC demonstrated MSHA patterns while the majority (75%) of the LT/ST producing ETEC demonstrated MRHA patterns (Table II).

Table I – Toxin production in 21 ETEC isolated from children with diarrhoea at the University Hospital

No. (%) of isolates producing toxin			Total
ST	LT	LT/ST	
15 (71%)	2 (10%)	4 (19%)	21

Table II – Haemagglutination patterns of the 21 CFA-negative ETEC isolates

No. of Isolates	Toxin Profile	Haemagglutination of erythroctes		
		Rabbit	Sheep	Human Group A
2	ST^+	MSHA[a]		MSHA
2	ST^+	MSHA		
1	LT^+/ST^+			MRHA[b]
1	LT^+/ST^+	MRHA		MRHA
1	LT^+/ST^+			MRHA
1	LT^+/ST^+		MRHA	
2	LT^+	-	-	-
11	ST^+	-	-	-

a - Mannose-sensitive haemagglutination
b - Mannose-resistant haemagglutination

DISCUSSION

To date, ETEC is reported to be one of the most common aetiological agents of diarrhoea with incidence rates ranging from 10% - 50% of total diarrhoeal cases[8]. In this study, ETEC was identified in 18% of the 62 diarrhoeal cases, with ST producing ETEC being most prevalent (71%). Similar prevalence patterns have been observed in studies on ETEC carried out in Bangladesh[15] and Argentina[1]. However, surveys on ETEC strains in various parts of the world have shown wide variations in the number of ETEC with adhesion factors.

Although CFA I, CFA II and CFA IV are found to be more commonly expressed, it has been reported that in comparison to surveys in Bangladesh, surveys carried out in South East Asia have yielded a far lower percentage of ETEC carrying either CFA I, CFA II or CFA IV[16]. In this study, interestingly it was found that ETEC isolated from Malaysian diarrhoeal cases did not seem to share similiar CFAs to that isolated from other neighbouring countries within the region. A reason for this discrepancy may be due to loss of CFA encoding plasmids which has been reported to be possible amongst clinical isolates of ETEC[15]. However, it seems unlikely for this phenomenon to occur in all the 21 ETEC isolates since the assays were carried out on fresh isolates. Therefore, it may indeed be possible that different putative CFAs yet unidentified, may prevail locally.

Binztein and co-workers[1] found in a study carried out in Argentina that 52 out of 109 (47.7%) ETEC strains were CFA-negative but amongst these strains, 24 (46.2%) produced MRHA using various erythrocytes. Similarly, in this study, the 21 CFA-negative ETEC isolates were tested for the presence of haemagglutinins and it was observed that 8 out of the 21 isolates presented differing HA patterns. Of the 15 ST-positive ETEC, only 4 were HA-positive and they demonstrated MSHA. In

comparison, all the 4 LT/ST-positive ETEC were HA-positive, with the majority (75%) demonstrating MRHA.

The different HA patterns observed amongst the different toxotypes support the possibility of the existence of other new putative colonisation factors, the identification of which would require additional screening methods. The absence of haemagglutination amongst the remaining 13 (62%) ETEC could be due to the loss of CFA encoding genes or due to the presence of other adhesins not associated with haemagglutination[1].

Therefore, further work is needed on these undefined colonisation factors, in order to increase our knowledge on the distribution and prevalence of colonisation factors of ETEC in this region and also to produce effective vaccines in this region as well.

ACKNOWLEDGEMENTS

We thank Professor Ann-Mari Svennerholm, University of Goteborg, Sweden for kindly providing us with the control strains and anti-CFA monoclonal antibodies. We also thank Miss Noriza Osman of the Bacteriology Diagnostic Unit for her assistance in the study. Finally, we thank the Malaysian Government Research Grant IRPA No. 3/075/01 for funding this research project.

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