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CHARACTERIZATION OF VEROTOXIN-PRODUCING STRAINS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) FROM CHILDREN WITH DIARRHOEA: EFFECTS OF THE TOXIN ON RABBIT INTESTINE

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ABSTRACT

249 enteropathogenic *Escherichia coli* (EPEC) strains from faecal samples of 90 children with diarrhoea were examined for verotoxin (VT) production. Polymyxin B extracts of 61 strains belonging to 9 serogroups from 35 children produced various verotoxins (VTs). The titre of these various VTs ranged from 1:40 to 1:1280.

Of the 26 (43.6%) adherent strains, 19 (73%) exhibited localized adherence (LA) and 7 (27%) manifested enteroadherent-aggregative (EA-Agg) patterns. The remaining 35 strains were nonadherent. Verotoxin producing EPECs elicited diverse effects on rabbit intestine ranging from no effect to severe damage to epithelial architecture, irrespective of their adherence patterns of HEp-2 cells. In conclusion, as far as EPEC pathogenesis is concerned, the role of verotoxins could not be established, but strains producing moderate to high levels of verotoxins may have an increased pathogenicity over non-producers.

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Keywords: Verotoxins, Enterotoxins, EPEC, HEp-2.

INTRODUCTION

Culture filtrates of some *E.coli* strains including EPEC have been reported to produce a cytotoxic factor active on vero cells. This factor was named verotoxin,[1] with subsequent reports from various regions describing the same phenomenon.[2-5]

E.coli isolates from hemorrhagic colitis[6] and hemolytic uremic syndrome[7] too are known to produce verotoxins/ Shiga-like toxins (SLT). To date, three distinct verotoxins. VT1 (SLT-I), VT2 (SLT-II), and VTe (SLT-II variant or SLT-IIv) have been identified and characterized.[8]

Epidemiological studies have shown the association of verotoxigenic *E.coli* (VTEC) with disease, whereas the experimental evidence so far although highly suggestive has not been conclusive.[9] The present study was conducted to assess the production of VTs by EPEC strains and the effect of the toxins on rabbit intestine. These strains were also further characterized by their adherence properties.

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MATERIALS AND METHODS

Bacterial strains

249 EPEC strains from faecal samples of 90 infants and children from birth to 12 years of age were used in this study. The EPEC strains were identified by slide agglutination using commercially available antisera (Wellcome Laboratories, U.K.). Control strains were *E.coli* ATCC 25922 (VT), *E.coli* H: 30 (VT) (provided by Dr. M.A.Karmali). *E.coli* 0148 (B7a) K?: H28 (LT, ST), *E.coli*

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K-12. F. Lac. Nal (non-adherent) and *E.coli* 2348/69(LA) (provided by Dr. J.B. Kaper).

Verotoxin production

The polymyxin-B extraction method[10] was used for the production and vero cells were employed for detection of the toxin.[1] VTs were heated at 70°C for 30 minutes and neutralization studies were done by incubating toxin-antitoxin mixture for 1 hour at 37°C followed by overnight incubation at 4°C prior to assay. Antitoxin, anti-VT 1 and anti-VT 2 were donated by Dr. G.B. Nair. Toxins were titrated by serial two-fold dilution. 0.1 ml of the diluted toxin was reassayed on Vero cells. The highest dilution value giving 50% cytotoxicity of the cells at the end of four days was considered as the titre of the toxin.

Heat-labile enterotoxin (LT)

Sterile culture supernates of EPEC strains grown with shaking in trypticase soya broth supplemented with yeast extract for 18h at 37°C, were tested on Chinese hamster ovary[5] (CHO) cells for the detection of LT.[11] or cytolethal distending toxin (CLDT).[12]

HEp-2 cell adherence assay

The procedure of Scaletsky et al.[13] was followed for the detection of various patterns. Localized adherence (LA) is a pattern in which the bacteria form microcolonies attached to HEp-2 cells at one or more places. In diffuse adherence (DA) bacteria are scattered all over the cell surface. Enteroadherent aggregative strains (EA-Agg) exhibit a stacked-brick pattern on the surface of the cells and the cover slip.

Animal model assays

Infant mice[14] and rabbit ileal loop assays[15] were used for the detection of heat-stable (ST) and heat-labile (LT) enterotoxins. Ligated intestinal ileal loops injected with live cultures of the verotoxin producing EPEC strains as well as the toxin preparation, were sectioned, stained with hematoxylin-eosin and visualized using a light microscope.

RESULTS

Verotoxin producing EPEC strains

Of 249 EPEC strains isolated from diarrhoeal cases, 61 (24.4%) produced VT. The effect on Vero cells was characterized initially by rounding and shrivelling of the cells, resulting finally in the disruption of the monolayer with the dead cells floating in the medium (Figs. 1-3). The cytotoxic factor was heat-labile. The VT-producing strains belonged to 9 different serogroups, and only strains of serogroups 044 and 086 were non-producers (Table I).

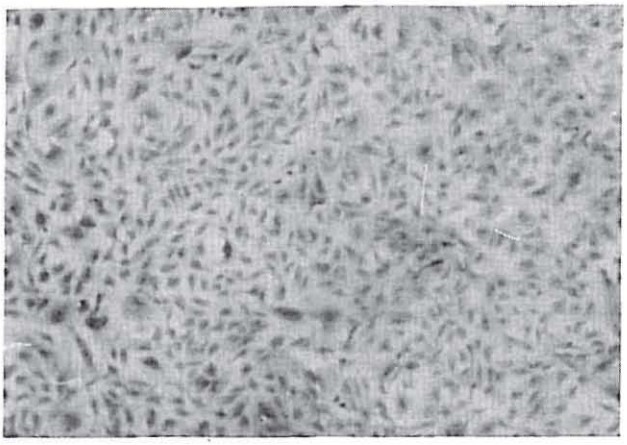

Fig. 1. Vero cells, normal monolayer.

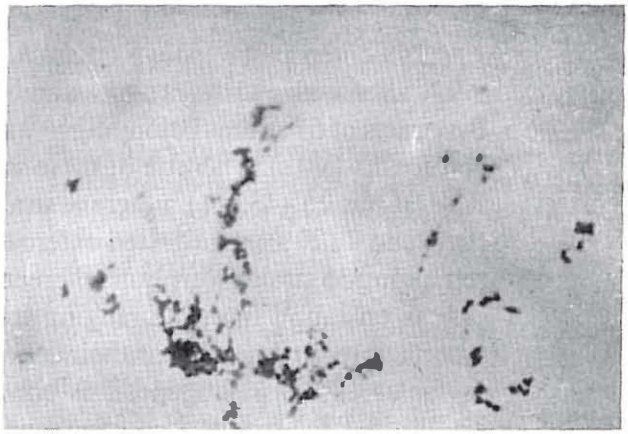

Fig. 2. Vero cells, effect of VT produced by *E.coli* H:30 (VT) .

Fig. 3. Vero cells, effect of VT produced by EPECs.

Neutralization experiments

Neutralization experiments carried out using anti-VT1 and anti-VT2, revealed that VT1 was produced by 41 (67.2%) strains with the titre of the toxin varying from 1:40

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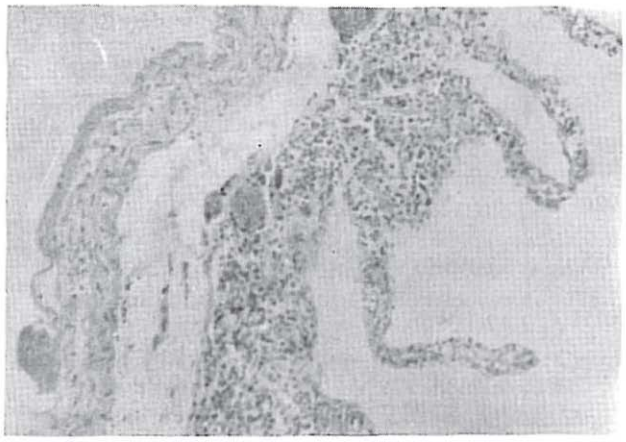

Fig. 4. Rabbit ileal segment injected with live culture of VT (non-neutralizable, low producer, EA-Agg).Section showing normal features except for some exudates.

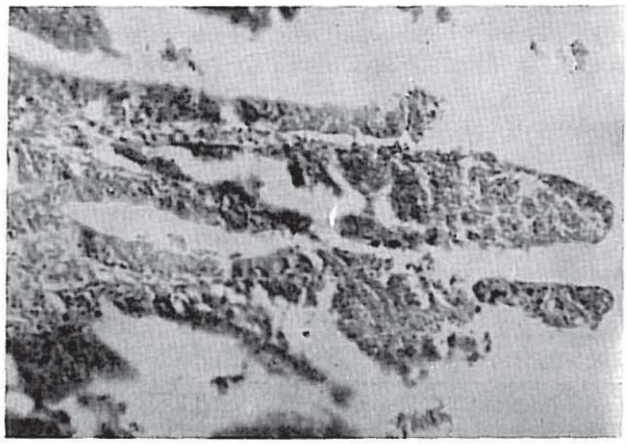

Fig. 5. Rabbit ileal segment injected with live culture of VT (cross-reactive, low producer, non-Ad). Section showing mild changes.

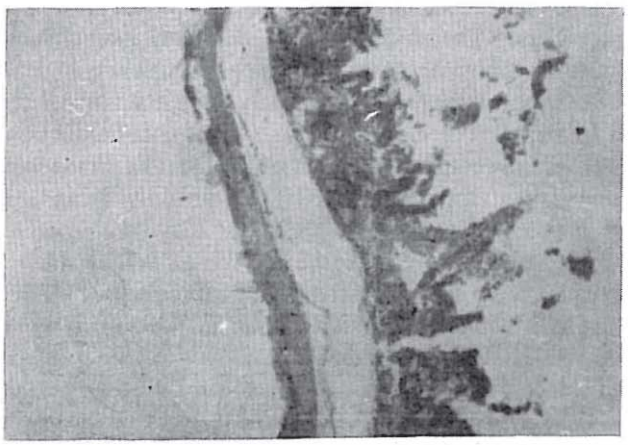

Fig. 6. Rabbit ileal segment injected with polymyxin extract of VT (VT1, high producer, non-Ad). Section showing severe changes.

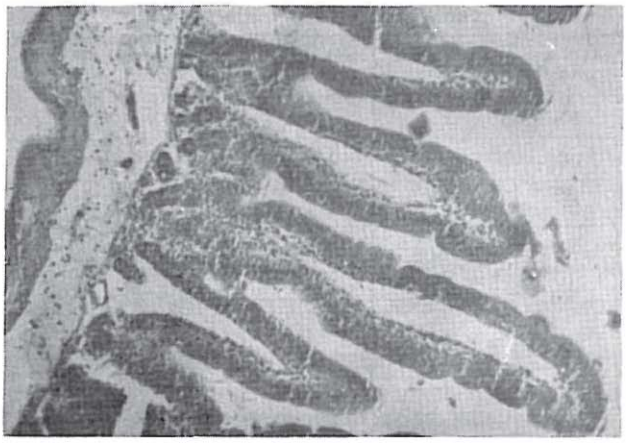

Fig. 7. Rabbit ileal segment injected with polymyxin extract of VT (VT2, moderate producer, LA). Section showing moderate changes.

to 1:1280. On the basis of the titre values, strains were classified as low (1:40, 1:80), moderate (1:160, 1:320) and high (1:640, 1:1280) VT-producers. According to this criterion, six strains were high VT1 producers, six strains were moderate and the remaining 29 strains were low VT1 producers. VT2 was produced in moderate levels hy five strains (8.2%).

Cross-reactive VTs in which verotoxic activity is neutralized by a mixture of anti-VT1 and anti-VT2, were produced by five (8.2%) strains and 10 (16.4%) strains produced low level VT which was not neutralized by either antitoxin alone or their mixture (Table II). Simuhaneous production of VTs and ST occurred in two strains only and production of VTs along with LT and/or CLDT was not observed.

Adherence property of verotoxin producing EPECs

Of the 61 verotoxin-producing EPEC, 26(42.6%) strains adhered to HEp-2 cells and the remaining 35 were nonadherent. Localized adherence (LA) was shown by 19(19/26= 73%) strains and enteroadherent-aggregative patterns (EA-Agg) were exhibited by 7(7/26=27%) strains (Table I).

Rabbit ileal assay

Theenterotoxic activity oflive culture and polymyxin B extracts of VT-producing strains of EPEC was assessed in rabbit ileal loop assay. All preparations except those from strains producing non-neutralizable verotoxic effects elicited enterotoxic activity of varying degree.

Histopathological features

Livecultures of VT and non-neutralizable toxin producers irrespective of their adherence pattern caused no abnormalities when stained sections of ileal loop injected with these strains were studied (Fig. 4), whereas VT2

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Table I: Verotoxin-producing EPEC

EPEC sero-groups	No. of strains tested	No. of VT strains	Adherence pattern of VT
026	3	2	Non-Ad
044	11	--	--
055	11	2	2LA
086	16	--	--
0111	10	2	2LA
0114	20	9	3 LA,6 Non-Ad
0119	7	4	2 LA,2 EA-Agg
0125	22	5	2 EA-Agg,3 Non-Ad
0126	47	6	1 LA,5 Non-Ad
0127	57	16	4 LA,1EA-Agg, 11Non-Ad
0128	45	15	5 LA, 2EA-Agg, 8Non-Ad
Total	249	61(24.4%)	--

LA= Localized Adherence
EA-Agg= Enteroadherent aggregative
Non-Ad= Non Adherent

producers and cross-reactive strains induced mild changes (Fig. 5). On the other hand, polymyxin extracts of the VT1 producers induced mostly severe (Fig. 6) and VT2 caused moderate changes (Fig. 7).

DISCUSSION

Production of verotoxin by EPEC strains appeared to be an inconsistent phenomenon both quantitatively and qualitatively. Toxin production was variable (titre values 1:40-1: 1280), and although the cytopathic effects on Vero cells correlated with the amount of toxin produced, such a correlation was not observed in *in vivo* experiments. Neutralization experiments revealed the production of different VTs by EPEC strains, and the use of cell lysate for this detection instead of culture supernate seemed more appropriate since the cell-bound VTs[16] could thus be recognized. Difficulty in detection of cross-reactive VTs which is due to the presence of both VT1 and VT2 toxins in the same preparation has also been reported by other investigators.[9] Non-neutralizable verotoxic effects were produced by some strains; such effects could be due to non-specific cytotoxic activity detectable because of the Vero cell line. It is important to note that all these strains were low VT producers.

No correlation was observed between various adherence patterns and verotoxin production by EPEC strains, suggestive of the independence of these two phenotypic traits. Similar observations have also been reported by other

Table II. Details of various VTs produced by EPECs.

	No. of VT producing strains(Titre)			
EPECs	VT1	VT2	cross-reactive VTs*	non-neutralizable VTs†
026	2(1:1280)	--	--	--
055	2(1:640)	--	--	--
0111	2(1:320)	--	--	--
0114	6(1:40, 1:80,1:320)	3(1:160)	--	--
0119	3(1:160)	--	--	1(1:40)
0125	2(1:80)	--	3(1:80)	--
0126	6(1:80, 1:160,1:640, 1:1280)	--	--	--
0127	11(1:40, 1:80,1:1280)	--	--	5(1:40,1:80)
0128	7(1:40, 1:80,1:160, 1:320,1:640)	2(1:320)	2(1:160)	4(1:40)
Total	41(67.2%)	5(8.2%)	5(8.2%)	10(16.4%)

*cross-reactive VTs= Verotoxic effect neutralizable by mixture of antitoxin (VT1+VT2)
†Non-neutralizable VTs= Verotoxic effects non-neutralizable by either antitoxin alone or their mixture.

investigators.[17,18] On the basis of quantitative determination, VTECs have been classified as high, moderate, and low producers.[19] The epidemiological data only incriminates moderate to high verotoxin producers as possible aetiological agents,[9] and in the present study only some strains met such a criterion. Therefore, although cytotoxin production undoubtedly confers additional advantages to a pathogenic organism, it seems that it is not the only means of pathogenicity.

Cattle are believed to be the reservoir of VTEC,[20] thus to obtain a more comprehensive picture of the role of VTEC and its possible transmission from animals to man, a more detailed study would be desirable.

In conclusion, although the role of VTs in EPEC pathogenesis could not be established, nevertheless an accessory role for these cytotoxins seems feasible in a multiple pathogenic system.

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