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Detection of Enterotoxigenic *Escherichia coli* isolated from Diarrhoeic Calves in Khartoum State, Sudan

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ملخص البحث

تم إجراء اختبارين لتقسيم معزولات بكتيريا الإشريكية القولونية إلى مجموعتين: مولدة للزيفان المعوي (entrotoxigenic) ومولدة للزيفان في خلايا القردة (Verotoxigenic). حددت الزيفانية للزنبانات المقاومة للحرارة (STa) والمتغيرة بالحرارة (LT) بإستخدام إختبار الفئران الرضيعة وفحص الطبقة وحيدة خلية القردة (verocell monolayer) لعدد أربع عشرة و أربع وثلاثين معزوله على التوالي. اثنا عشرة معزوله أعطت نتائج موجبة فى الفئران الرضيعه وعشر فقط كانت موجبة لمقايسة خلايا القردة.

Summary

For grouping of *E.coli* isolates into enterotoxigenic and verotoxigenic; two characterization tests were performed. These were the suckling mice test and verocell monolayer assay. Fourteen isolates were examined by the suckling mice test, twelve were positive i.e. enterotoxigenic. On the other hand, ten out of 34 *E. coli* isolates assayed by the verocell monolayer were positive.

Introduction

Generally, there are two types of bacterial toxins namely endotoxin and exotoxin. The endotoxins are innate derivative components of Gram-negative bacterial cells while the exotoxins are all excreted, with few exceptions, by Gram-positive organisms; *Shigella dysenteriae* and V*ibrio cholera*, which are Gram-negative, produce exotoxins. Exotoxins are proteins, probably without any non-protein residues, and are antigenic. Endotoxins are antigenic complex of protein, polysaccharide and lipid; their protein, polysaccharide, and possibly some of the lipid determine their antigenicity, immunogenic specificity and toxicity, respectively (Van-Heyningen, 1970).

Several forms of enteric diseases are attributed to *Eschercia coli* infection (Moon, 1974). Enterotoxigenic *E. coli* (ETEC) strains are of particular concern as they cause neonatal diarrhoea in domestic animals and man (Tzipori, 1981). They cause diarrhoea in newborn piglets and calves (Gupta and Sigh, 1972; Guel and Malik, 1974) and pathogenic *E. coli* was isolated from 94% of faecal samples collected from calves less than three-month-old (Perez *et al*., 1998).

The virulence factors of *E. coli* include fimbriae (adhesins) and production of enterotoxins (Levine *et al*, 1983; Evans *et al*, 1984; Levine, 1987). *E. coli* strains that cause diarrhoea have recently been classified into six groups according to their pathogenesis and their corresponding clinical manifestations; among these is ETEC (Albert *et al*, 1995; Okeke *et al*, 2000). These strains cause acute watery diarrhoea by colonizing the small intestine, where they produce one or both of two enterotoxins; heat-labile (LT) and heat-stable (STa) toxin. The STa enterotoxin is unaffected by temperature of 60^{o}C for 30 min. and occurs mainly within the bacterial cell). ETEC strains cause colibacillosis in the youngs (Gyles, 1993).

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The oral-faecal route is likely the most portal route of infection in calves, piglets and lambs. The major virulent factors of ETEC in calves are the $K99^+$ adhesive antigen and the STa enterotoxin (Mainil *et al*, 1990; Quinn *et al*, 2001). The common enterotoxin of almost all strains of *E. coli* that cause diarrhoea in calves, referred to as STa, is composed of 18 amino acids with 6 cysteine residues. This enterotoxin is pathogenic and is also present in strains that infect other hosts including man. The STa enterotoxin from ETEC has been purified from bovine isolates and characterized (Saeed *et al*, 1983).

The objective of this study was to determine potentially pathogenic *E. coli* strains isolated from diarrhoeic calves in Khartoum State, Sudan with special reference to their being enterotoxigenic or verotoxigenic strains, and hence ascertain their roles in causing diarrhoea.

Materials and Methods

This investigation was mainly carried out in Khartoum State, Sudan, during the period of 1998 – 2002. Three to five ml of watery faeces were collected from each of 1-21-day-old diarrhoeic calf, that had not been treated with antibiotics.

Suckling Mice Test (SMT):

Toxin extraction:

Two ml of the test culture were inoculated into 100 ml Tryptone Soya Broth (Oxoid, CM129) in 250-ml Erlenmeyer flask and incubated overnight at 37^{o}C with continious shaking at 120 rpm. The culture was centrifuged at 4000 rpm for 30 mins at 4^{o}C and the supernatant was aseptically collected and used for inoculation of suckling mice. The mice were obtained from the Experimental Animals Facility, National Health Laboratory, Ministry of Health, Khartoum, Sudan.

The test procedure (SMT):

The test was carried out according to Dean *et al*. (1972). It depends on the fact that certain enterotoxin-producing *E. coli* cause fluid accumulation in the intestines of small animals (Moon and Whipp, 1971).

Two to four-day-old infant mice were collected immediately after suckling and grouped randomly in groups of three each. They were directly inoculated into the stomach, which should be full of milk, with 0.1 ml of the test material via a special canula. Thereafter, they were kept at 30^{o}C for 6 hours before being euthanized with chloroform inhalation.

The abdomen was opened, using sterilized dissection set, and the small intestines were examined *in situ* for distension before they were removed with sterile forceps. The intestines of each mouse were weighed separately as well as the remaining carcass of the mouse. The ratio of the intestine weight to that of the remaining carcass weight for each mouse was calculated and finally the mean ratio for each group was determined. Ratios of less than 0.070 were considered negative; those ranging between 0.07-0.09 were considered doubtful and those over 0.09 were considered positive. Fourteen isolates of *E. coli* were tested by the suckling mice test, viz isolates nos. 1 to 14.

Verocell monolayer test:

Preparation of culture supernatant:

Single colonies were plated out onto Blood Agar (Oxoid) and incubated overnight at 37^{o}C. Subcultures into Tryptone Soya Broth (Oxoid) were thereafter incubated overnight at 37^{o}C. Polymyxin B (10,000 units/ml) was added and the broth was incubated for a further 4 hrs on orbital shaker. The cultures were then centrifuged at

4000 rpm for 30 min. at 4^{o}C before the supernatants were filtered through a pore size of 0.22 µm.

The test:

Verocells of three days passage were harvested; they were re-suspended in Eagle's Medium following trypsine versenes treatment to give 2x10^{5} cell /ml. A 96-well tissue culture tray was seeded with 200 µl of the cells suspension and incubated at 37^{o}C under 5% carbon dioxide and relative humidity for 18-20 hours.

The outer rows of wells were not used and every other well was left as a negative control, for ease of comparison. A volume of 10 µl of the polymyxin B extracts from cultures of *E. coli* isolates were each added to a test well. The plates were covered and incubated at 37^{o}C under 5% carbon dioxide and raised relative humidity for 24-72 hr. Culture supernatants of 34 *E. coli* isolates from diarrhoeic calves were each inoculated onto a verocell monolayer.

Results

Suckling Mice Test (SMT):

Fourteen *E. coli* isolates were examined for their production of a heat-stable toxin (STa). The weight of intestine, weight of remaining carcass and calculated mean ratios for the test isolates are shown in Table 1. A mean ratio of 0.09 or over (>0.09) was considered indicative of presence of STa, i.e. positive result. On the other hand, a ratio of less than 0.07 indicated a negative result. Twelve out of the 14 (85.7%) *E. coli* isolates examined produced STa and only two were negative (14.3%).

Verocell monolayer assay:

The test for LT was read at 24 hrs for cytotoxic necrotizing factor. LT causes cells to lose their elongated appearance and tend to become round or globular. Effect of the cytonecrotizing factor was demonstrated by changes, which occured at 24-48 hr of incubation. It tended to make the affected cells enlarge and have a multinucleated appearance.

Production of a heat-labile toxin (LT) was indicated by a round morphology of the inoculated Vero cells (shape change from long to round). Accordingly, ten *E. coli* isolates gave positive results, i.e. verotoxigenic, in 24 hr period of incubation.

Discussion

Enterotoxigenic colibacillosis is referred to the form of diarrhoea which is characterized by enteric toxaemia. A characteristic, age-specific prevalence rate of the pathogen is observed; ETEC is most frequently isolated from calves within the first week of age and rotavirus in the second week. ETEC and rotavirus cause the majority of acute cases of diarrhoea in piglets, calves and infants (Smith and Hall, 1967a). An ETEC strain has the ability of producing an enterotoxin that causes an increase in net secretion of fluid and electrolytes from the systemic circulation into the lumen of the intestine (Smith and Hall, 1967b; Burgess *et al,* 1978; Frantz and Robertson, 1981; Guerrant, 1990).

Table 1: Weight of intestine, residual carcass and calculated mean ratio of suckling mice used in SMT for detection of STa toxin produced by *E. coli* isolated from diarrheic calves.

Isolate No.	Int. 1	Carc. 1	Ratio	Int. 2	Carc. 2	Ratio	Int. 3	Carc. 3	Ratio	Mean int.	Mean carc.	Mean ratio
1	0.69	4.65	0.148387	0.65	4.43	0.146727	0.69	4.59	0.150327	0.676667	4.556667	0.1485
2	0.67	4.45	0.150562	0.36	3.46	0.104046	0.68	4.41	0.154195	0.57	4.106667	0.138799
3	0.63	4.33	0.145497	0.66	4.40	0.1500	0.68	4.39	0.154897	0.656667	4.373333	0.150152
4	0.64	4.14	0.154589	0.66	4.64	0.142241	0.67	4.74	0.14135	0.656667	4.506667	0.14571
5	0.66	4.82	0.136929	0.65	5.17	0.125725	0.68	7.80	0.087179	0.663333	5.93	0.111861
6	0.96	6.52	0.147239	1.03	6.77	0.152142	0.66	5.19	0.127168	0.883333	6.16	0.143398
7	0.92	6.52	0.141104	0.89	5.99	0.148581	0.82	4.35	0.188506	0.876667	5.62	0.155991
8	1.03	8.77	0.117446	1.31	10.18	0.128684	1.18	10.72	0.110075	1.173333	9.89	0.118638
9	0.71	7.22	0.098338	0.70	6.19	0.113086	0.69	6.82	0.101173	0.7	6.743333	0.103806
10	0.43	6.64	0.064759	0.41	6.81	0.060206	0.42	7.10	0.059155	0.42	6.85	0.061314
11	0.41	6.81	0.060206	0.39	6.11	0.06383	0.44	6.81	0.064611	0.413333	6.576667	0.062848
12	1.31	9.95	0.131658	0.66	10.57	0.062441	D	D	D	0.985	10.26	0.096004
13	0.65	7.80	0.083333	0.68	10.81	0.062905	0.96	6.52	0.147239	0.763333	8.376667	0.091126
14	1.03	8.77	0.117446	0.91	9.27	0.098166	0.86	10.11	0.085064	0.933333	9.383333	0.099467

D=Died int= intestines carc= carcass; SMT= Suckling mice test.

Pathogenic *E. coli* could not be separated from non-pathogenic one on the basis of their biochemical characteristics, as there is no biochemical marker that distinguishes them from one another. Therefore, to determine the pathogenic potential of an isolate of *E. coli* one or more of the pathogenicity tests should be carried out (DuPont *et al*, 1971; Dean *et al*, 1972; Guerrant *et al*, 1974; Gyles, 1974; Sack, 1975; Giannella, 1976). One of the approaches is SMT. It is cheap, fast and gives reliable consistent results (Andrew *et al*, 1972). Fourteen isolates were tested by the SMT in this study, for detection of the heat-stable (STa) toxin. Twelve isolates were positive (85.7%). The reliability of the SMT for detection of STa toxin production by *E. coli* was also confirmed by Andrew *et al*. (1972). In a report by Dean *et al* (1972), six human and one porcine *E. coli* isolates known to have produced positive results in rabbit loops, had also strongly dilated the intestine of the infant mouse. The SMT test used is preferable to ligated ileal segment of adult rabbit because of the initial absence of bacteria in infant mice intestines, their high degree of uniformity and their small size. Moreover, the ligated ileal segment assay is expensive, time consuming and sometimes gives variable results (Andrew *et al*, 1972).

The most important factors in the initiation of colibacillosis are the immune status of the animal and the properties of the strain of *E. coli* particularly its capacity to invade tissues and produce septicaemia or an entertoxin which causes varying degrees of diarrhoea. This enterotoxin causes increase in the flow of net secretion of fluids and electrolytes from the systemic circulation into the lumen of the gut and none of the entropathogenic strains adhere to the intestinal epithelium (Hadad and Gyles, 1982). There are great variations among different strains of the same bacterial species in their capacity to produce toxins on a given culture medium, even if it is optimally prepared and incubated. On repeated subculture, most toxigenic strains of *E. coli* lose their ability to produce toxins (Van Heyningen, 1970).

For toxin production and purification it is better to take advantage of the high yields that can be obtained from "rich" complex culture media. To promote good growth a number of factors such as pH, organic and inorganic constituents and the gas phase must be controlled. However, toxin produced by a toxigenic bacterial strain in suitable culture medium may be affected by some factors, eg physical conditions such as the volume of the culture medium, its aeration and agitation. The yield of toxin was found to vary from one culture medium to another (Starvic *et al*, 1978; Bertschinger *et al*, 1990), and it is uncertain that the media recommended will produce the highest yield of STa from bovine ETEC (Saeed *et al*, 1983).

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