Production of *Escherichia coli* Heat-Labile Enterotoxin (LT) in Some Artificial Media and Commercially Available Baby Foods

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Abstract

The production of thermolabile (LT) enterotoxin on some artificial media and commercially available baby foods was studied. Staples medium, Casamino acids-Yeast extract (CA-YE) medium, Tryticase soy broth (TSB) and Syncase medium were evaluated for their ability to support the production of LT-toxin. Also, nine commercially available baby foods were evaluated as substrates for LT-toxin production. Rabbit toxicity indices (RTI) showed that Staples medium served as the best artificial medium for toxin production (RTI = 1.9) followed by CA-YE (RTI = 1.2). Three (NA, CER, and BOC), out of nine baby food brands examined supported LT-toxin production with NA giving the highest yield of toxin (RTI = 2.2). Determination of the electrolyte content of aspirated diarrhoeal fluid showed significant secretion of sodium (Na^+) and potassium (K^+) electrolytes into the fluid. These results suggest that some baby foods, when prepared and stored under non-hygienic conditions could permit growth of, as well as toxin production by toxin-producing bacteria and pose a health hazard for the infant population.

Keywords: *Escherichia coli,* thermolabile enterotoxin (LT); baby foods; artificial media

Introduction

Enterotoxigenic *E. coli* (ETEC) is a common cause of diarrhoea in children in developing countries (Levine *et al.*, 1993; Okeke *et al.*, 2000; Oronsaye and Oziegbe, 2002). Most diarrhoeal episodes occur in the first two years of life with highest incidence in the age group 6 – 11 months, during weaning (Gomes *et al.*, 1991). In these circumstances, the risk of developing severe diarrhoea is many times greater in non-breastfed infants than in infants who are exclusively breastfed (World Health Organization Food Safety Unit, 1993; Oronsaye and Oziegbe, 2002). This epidemiologic pattern reflects the combined effects of declining levels of maternally acquired antibodies, the lack of active immunity in the infant, direct contact with environment contaminated by faecal matter as the infant starts to crawl, and the introduction of food that may be contaminated with enterotoxigenic bacteria (Gomes *et* al., 1991; Fleisher and Ludwig, 1993).

Studies have shown that many outbreaks of ETEC-induced diarrhoea are related to contaminated foods, and among the foods, dairy products may serve not only as incidental vectors but also sometimes as media for enterotoxin production (Turnbull, 1979).

There are two types of enterotoxin produced by ETEC – the heat-stable toxin (ST) and heat-labile toxin (LT). Some studies have suggested that the production of one or the other or both toxins is dependent on conditions of growth of the *E. coli* strain (Alderete and Robertson, 1977; Staples *et al.*, 1980; Fontes *et al.*, 1982).

Enterotoxigenic *E. coli* is an important cause of severe and often life-threatening diarrhoea among infants in developing countries, including Nigeria, and it has been associated with weaning. Commercial baby foods are important components of infants' diets worldwide, particularly during weaning. This study was undertaken to evaluate the relative capacity of some commercially available baby foods to support LT-toxin production by ETEC in comparison with some chemically defined bacteriological media.

Materials and Methods

Organism: The *Escherichia coli* strain used in this study was generously provided by Dr. Mulindwa of the Institute of Veterinary Medicine, Robert Von Ostertag Institute, FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses, West Berlin, Germany. The strain is a known producer of heat-labile enterotoxin (LT).

Animals used: Rabbits aged between nine and twelve weeks were purchased from the Amawbia Rabbitry and maintained for two weeks in the Animal House of the Faculty of Biological Sciences, University of Nigeria, Nsukka, before use.

Media preparation: Four different media made up of Difco's Trypticase soy broth (TSB) and three synthetic media were used. The synthetic media were Casamino acids – yeast extract medium (CA-YE), Syncase medium and Staples medium; and were constituted as follows (g/L):

CA-YE - Casamino acid, 20.00; Yeast extract, 1.50; NaCl, 2.50; K_2HPO_4, 8.71; $MgSO_4$, 0.05; $FeCl_3$, 0.005, and $MnCl_2$, 0.05.

Syncase - Na_2HPO_4, 5.00; K_2HPO_4, 5.00; Sucrose, 5.00; NH_4Cl, 1.18; Na_2SO_4, 0.089; $MgCl_2.6H_2O$, 0.042; $MnCl_2.4H_2O$, 0.0042; $FeCl_2.6H_2O$, 0.005; acid hydrolyzed casein, 10.00.

Staples - NaCl, 2.52; sodium acetate, 10.00; $K_2HPO_4.3H_2O$, 11.42; asparagine, 5.00; Na_2SO_4, 0.14; $MgSO_4$, 0.05; $MnCl_2$ (5µg) and $FeSO_4$ (5µg).

The $MnCl_2$ and $FeSO_4$ were first prepared as 0.5% ($^w/_v$) stock solutions. One milliliter of each stock solution (0.005g/ml) was then added to a solution of the other salts and made up to the 1 litre mark.

Commercial baby foods: Nine commercially available baby foods were used in the study. Details of the brand names and identity of the food manufacturers have been withheld on legal grounds and codes assigned to the different foods. The code names and major contents (%/100g Powder) of the various foods are as follows:

BOC – Corn starch, artificially coloured and flavoured.

CER – Protein, 11.0; Fat, 7.8; Carbohydrate, 77.7; Mineral salts, 2.0; Moisture, 1.5; Vitamins A, B_1, B_2, B_6, B_{12}, C, D, E and Calcium, 0.2 – 20mg; Folic acid, 12µg.

FAC – Protein, 18.0; Fat, 10.0; Carbohydrate, 64.4; Minerals, 3.9; Moisture, 3.7; Vitamins A, B_1, B_2, C, D, Iron and Nicotinamide, 0.6 – 70mg; Calcium, 760mg.

FAN – Protein, 20.0; Fat, 4.5; Carbohydrates, 67.0; Minerals, 3.5; Moisture, 5.0; Vitamins A, B_1, B_2, C, D, Iron and Nicotinamide, 0.6 – 70mg, Calcium, 780mg.

LAT – Protein, 21.6; Fat, 1.0; Carbohydrates, 51.6; Minerals, 4.8; Moisture, 3.0; Vitamins A, B_1, B_2, B_6, C, D, E, Iron, and Niacin, 0.2 – 37mg; Calcium, 770mg.

NA – Protein, 12.5; Fat, 26.0; Carbohydrate, 56.2; Ash, 2.3; Moisture, 3.0; Linolate, 3.3; Vitamins A, B_1, B_2, B_6, C, D, E, Iron and Niacin, 0.3 – 41mg; Calcium, 340mg.

SIL – Protein, 13.0; Fat, 8.0; Carbohydrates, 72.0; Minerals, 2.5; Moisture, 4.5; Vitamins A, C, D, E and Iron, 0.2 – 36mg.

SAM - Protein, 11.9; Fat, 27.7; Carbohydrates, 55.4; Ash, 3.0; Moisture, 2.0; Vitamins A, B_1, B_2, B_6, C, D, E, Iron and Niacin, 0.4 – 58mg; Calcium, 560mg.

Akamu/Ogi – Locally produced corn gruel (exact contents not known).

Two concentrations (full strength and half strength) of each baby food were used. Full strength preparations were according to the recommendations of the manufacturers while half strength preparations (1:2 dilutions of the full strength concentrations) reflected the concentrations sometimes used by some mothers for economic reasons.

Culture in artificial media and baby foods: To 100ml volume of each artificial medium or baby food (reconstituted in sterile water) was added a 5ml inoculum of an 18-h brain heart infusion broth culture of the test organism. Incubation was as a stationary culture for 24h at 37°C, after which bacterial cells were harvested by centrifugation at 5000 rpm for 30 min. The harvested cells were resuspended in 5ml sterile distilled water and disrupted ultrasonically in a Gallenkamp ultrasonic disintegrator. Unbroken cells and large cellular debris were removed by centrifugation at 5000 rpm for 30 min in a

refrigerated centrifuge (Beckman Model J.21). The supernatant was then filtered through a Millipore filter of 0.22μm pore diameter and the filtrate was tested for LT-toxin activity by the Ligated Ileal Loop Assay.

Ligated ileal loop assay for LT-enterooxin: Ligated ileal loop assays were conducted according to the technique of Smith and Gyles (1970) using nine to ten week old rabbits. The animals were starved for 30h just before challenge, but water was supplied *ad libitum.*

Each rabbit was anaesthesized with ether and secured in dorsal recumbency. Following a midline incision, and starting 10cm from the pyloric end, the small intestine was divided into segments of 5cm in length, with string ligatures. The test materials (0.5ml) were injected into alternate segments leaving the intermediate as untreated controls. Uninoculated broth and a preparation from enterotoxigenic *E. coli* served as negative and positive controls respectively. The incision was then sutured and each animal allowed to recover from anaesthesia. After 14h, the animals were sacrificed and the segments examined for fluid accumulation (dilatation).

For positive loops, the volume of fluid recovered by aspiration was used to determine the rabbit toxicity index (RTI) by calculating the ratio of volume of fluid to length of loop. An RTI $\geq$ 0.2 was taken as positive. Each test was done in two different animals. To minimize variations due to differences in reactivity of individual portions of the gut, the positions of the loops receiving a given preparation were rotated in different animals.

Determination of electrolyte contents of diarrhoeal fluid: Fluid was aspirated into test tubes from loops showing positive reactions, using sterile syringe and analyzed for sodium (Na^+) and potassium (K^+) ions. The electrolytes were measured with a Gallenkamp flame photometer (FARTH GROUND). Where necessary, the fluid was diluted before measurement. Data generated were analysed by the Students' T-test.

Results and Discussion

Production of LT-toxin in artificial media: The mean toxicity indices obtained in the rabbit ileal loop tests showed evidence of LT-toxin production (RTI $\geq$ 0.2) in the four artificial media tested (Table 1). The highest RTI was recorded in Staples medium (RTI = 1.9). Although a similar observation was made by Fontes *et al* (1982), a number of other workers failed to confirm the superiority of Staples medium over the other artificial media as substrate for LT-toxin production and have variously reported adequate levels of toxin production from different media. For instance, Rivas *et al* (1987) reported Evans medium as the best medium for LT-production in their study while Stepanova *et al* (1985) reported very high yields of LT-enterotoxin (1.4 and 1.0mg/L of culture medium) for two different *E. coli* strains grown in a culture medium formulated and manufactured in the USSR. It appears that production of enterotoxins in artificial media may be dependent on the combined effects of a variety of factors such as addition of sugars, fatty acids, vitamins, antibiotics and incubation condition – stationary or shaking (Fontes *et al.*, 1982; Rivas *et al.*, 1987; Busque *et al.*, 1995).

Production of LT-toxin in commercial baby foods: Of the nine baby foods studied, three (NA, CER, and BOC) supported LT-enterotoxin production at both half-strength and full strength concentrations. The highest level of toxin (RTI = 2.24) was recorded in NA; closely followed by CER (Table 2). All three baby food brands gave higher yield of toxin at half-strength concentration.

It is difficult to draw a conclusion on why some baby foods favoured toxin production over others. There is a paucity of literature on baby foods as substrates for LT-toxin synthesis and it has been previously suggested that production of enterotoxins may be dependent on the combined effects of a variety of factors. There are also reports from a number of workers that some sugars such as glucose, lactose and sucrose are stimulatory to LT-enterotoxin production (Giligan and Robertson, 1979; Fontes *et al.*, 1982; Rivas *et al.*, 1987; Busque *et al.*, 1995). While there is no information on the exact sugars contained in the baby foods tested here, the manufacturers' labels and product information, however, show that all three formulations are rich in carbohydrate, fat and protein, in addition to having a good supplement of vitamins, particularly the B vitamins, as shown above (Methods).

Table 1: LT-Toxin Synthesis In Artificial Media

Medium	pH	Length of segment (cm)	Volume of fluid (ml)	V/L RTI	Result
Staples medium	7.50	5	9.5	1.9	+
Casamino acids yeast extract medium	7.30	5	6.0	1.2	+
Trypticase soy broth	6.90	5	5.5	1.1	+
Syncase medium	6.10	5	5.0	1.0	+
Control		5	0.5	0.1	-

RTI was determined by finding the ratio of the volume of fluid to the length of ileal loop segment. RTI ≥ 0.2 were adjudged positive while ratios < 0.2 were scored negative (according to Smith and Gyles, 1970).

Table 2: LT-Toxin Synthesis in Commercial Baby Foods

Medium (baby diet)	PH	Length of segment (cm)	Volume of fluid (ml)	V/L RTI	Result
Half-strength Formulation					
NA	5.35	5	11.2	2.24	+
CER	5.30	5	9.0	1.80	+
SAM	5.00	5	3.0	0.60	+
BOC	4.70	5	2.5	0.50	+
LAT	5.20	5	<0.1	<0.1	-
SIL	4.90	5	<0.1	<0.1	-
FAN	5.90	5	<0.1	<0.1	-
FAC	5.40	5	<0.1	<0.1	-
Akamu (ogi)	4.50	5	<0.1	<0.1	-
Positive control	6.90	5	7.0	1.4	+
Negative control		5	0.5	0.1	-
Full Strength					
NA	5.30	5	7.0	1.4	+
CER	5.25	5	6.5	1.3	+
BOC	5.00	5	2.5	0.5	+
SAM	5.15	5	<0.1	<0.1	-
Positive control	6.90	5	7.0	1.4	+
Negative control		5	0.5	0.1	-

RTI was determined by finding the ratio of the volume of fluid to the length of segment. RTI ≥ 0.2 were adjudged positive (+) while RTI less than 0.2 were marked negative (-) according to Smith and Gyles, 1970.

NA, which gave the highest yield of toxin, was also the only brand, which contained linolate. We can only conclude at this point that the three baby foods; NA, CER and BOC provided a more favourable mixture of nutrients for toxin production.

Electrolyte content of diarrhoeal fluid: The diarrhoeal impact of the LT-toxin from the baby foods on the rabbit ileum is shown in Figures 1 and 2. The fluid aspirated from the various positive ileal loops, showed

Table 3: Quantitative determination of the electrolyte (Na^+ and K^+) content of diarrhoeal fluid

MEDIUM	Na^+ (ppm)	K^+ (ppm)
Staples medium	3375	1950
Casamino acids-Yeast extract	3250	2150
Trypticase soy broth	4625	3500
Syncase medium	3125	2600
Commercial Baby Foods (Half Strength)		
NA	3750	2500
CER	4250	2750
SAM	3625	2800
BOC	5875	2350
Control	2875	500
Full Strength		
NA	2500	1100
CER	2000	1000
BOC	2375	1500
Positive control	2625	1500
Negative control	300	200

Fluid was aspirated from loops showing positive reactions and sodium (Na^+) and potassium (K^+) ion contents were measured with a Gallenkamp flame photometer (FARTH GROUND).

significant increase ($p < 0.05$) in their sodium (Na^+) and potassium (K^+) contents as compared with the control (Table 3), similar to what would be observed in diarrhoeal conditions. These observations have implied significance in view of the known consequences of electrolyte loss and particularly, potassium loss, which may lead to heart rhythm disturbances and ultimately heart failure in persons suffering from prolonged diarrhoea. Nevertheless, we must acknowledge that LT-enterotoxin production in baby foods similar to that described here is only probable under non-hygienic conditions of food handling and preparation, which permit high levels of *E. coli* contamination of the foods.

We must also acknowledge that the apparent higher production of LT-toxin (as measured by RTI) and the increased electrolyte loss with half-strength food preparation was surprising but interesting. We cannot at this point explain this occurrence except to speculate that at half-strength, the nutrient concentrations were perhaps more favourable for growth of the organism and toxin production. The results presented here raise some very interesting questions and call for further investigation.

Acknowledgments: The authors thank Dr. Mulindwa of the Institute of Veterinary Medicine, Robert Von Ostertag Institute, FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and

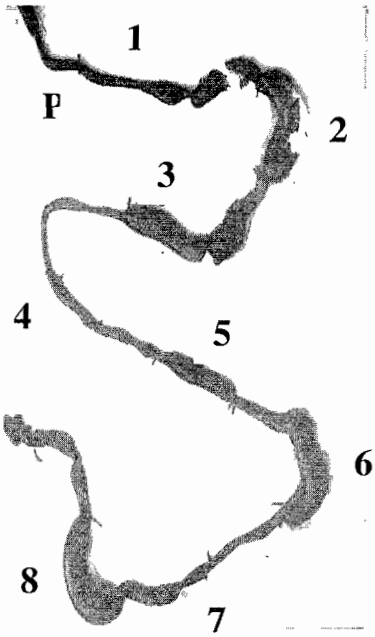

Fig. 1: Ligated segments of rabbit intestine after injection with LT-toxin derived from baby foods at half strength. The ligated sections were injected as previously described: p, pyloric end; 1, Farlac; 2, Cerelac; 3, Custard; 4, Akamu; 5, Lactogen; 6, Positive control; 7, negative control; and 8, Nan.

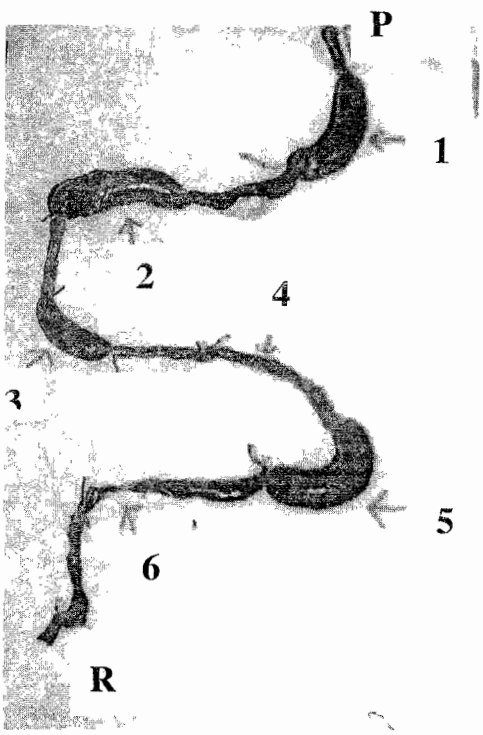

Fig 2: Ligated segments of rabbit intestine after injection with LT-toxin derived from baby foods at full strength. Injections were done as previously described: p, pyloric end; 1, Nan; 2, Cerelac; 3, Custard; 4, SMA; 5, positive control; 6, negative control; and R, rectal end.

Zoonoses, West Berlin, Germany, for providing the *Escherichia coli* strain used in this study; Professor S. M. Anika of Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, for assisting with the surgery on the animals, and Professor S. K. C. Obi of the Department of Microbiology, University of Nigeria, Nsukka, for critical reading and editing of this manuscript.

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