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Enteropathogen carriage by healthy individuals living in an area with poor sanitation

By G. FIGUEROA, M. TRONCOSO, M. ARAYA, J. ESPINOZA and O. BRUNSER

Institute of Nutrition and Food Technology (INTA), University of Chile, Casilla 15138, *Santiago* 11, *Chile*

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SUMMARY

Faecal carriage of bacterial enteropathogens (enteropathogenic *Escherichia coli* (EPEC), shigellae and salmonellae) was studied in 265 individuals: 65 infants 3–6 months of age (50 bottle-fed and 15 breast-fed), 100 school-age children 8–10 years of age and 100 adults 21–50 years of age. All were apparently healthy, did not have gastrointestinal symptoms, had not received antibiotics in the preceding fortnight and were not malnourished. Enteropathogens were isolated from the faeces of 24 individuals (9·1%). Cultures were positive for enteropathogens in 20% of the infants (both breast- and bottle-fed), 8% of school-age children and 3% of the adults. EPEC was the most frequent isolate. Twelve different serotypes were detected. The highest recoveries were *E. coli* 026:K60 and 044·K74. Shigella was detected only in school-age children (2%) and salmonella only in adults (1%). *Campylobacter jejuni* and *Yersinia enterocolitica* were studied only in the school-age children: there was one isolate of each of them. Most enteropathogens isolated were susceptible to the majority of the antibiotics tested. Only four *E. coli* strains, isolated from bottle-fed infants, could be considered multi-resistant. Two of the strains were *E. coli* 044:K74 and 020a020c:K61. The remainder were *E. coli* 0111:K58 and were capable of transferring some of their antibiotic resistance traits to a recipient strain.

INTRODUCTION

Carriage of bacterial enteropathogens is characterized by intermittent or continuous faecal excretion of these micro-organisms by an apparently healthy individual. The carrier state may arise from a previous episode of clinical disease (convalescent carrier) or from asymptomatic infection (healthy carrier). In both conditions faecal excretion of these bacteria does not necessarily occur in large numbers or continuously. This is probably related to characteristics of both the agent and the host. Carriers are reservoirs of potentially dangerous bacteria and contribute to their maintenance in the environment. Survival and spread of pathogens is also clearly related to factors such as sanitation, hygienic habits and other incidental factors such as the job held by the carrier (food handler, nurse, etc.). (Brush *et al.* 1963; Mata, 1978; Mata & Urrutia, 1971.)

Knowledge about carriage rates is important in the interpretation of enteropathogens in acute diarrhoea. This is most relevant in underdeveloped countries where faecal carriage of enteric pathogens is likely to be high (Mata, Catalan & Gordon, 1966; Mata, Fernández & Urrutia, 1969; Mata, 1978).

The objective of this study was to evaluate the carrier state for enteropathogens in apparently healthy individuals belonging to different age groups and living in an area with defective environmental sanitation. Plasmid-mediated resistance to antibiotics may be considered a virulence factor (Gangarosa *et al.* 1972). For this reason, the transfer of resistance plasmids by multi-resistant strains was also investigated.

MATERIALS AND METHODS

Subjects. Volunteers were selected who fulfilled the following requirements.

(*a*) They all belonged to the low socioeconomic level as demonstrated by a validated modification of Graffar's Scale (Alvarez, 1982).

(*b*) They had been free of gastrointestinal illness in the preceding fortnight.

(*c*) They had not received any antibiotic treatment during the same period.

(*d*) The nutritional status of individuals studied was within normal limits according to accepted standards (Frisancho, 1974; Jellife, 1968, N.C.H.S., 1977). Their physical examination did not disclose any sign of past or current malnutrition.

(*e*) None of the individuals studied had been hospitalized in the preceding month.

Individuals belonged to one of the following three groups: group A was formed by 65 infants, 3–6 months of age, who attended a 'well baby' clinic at a Health Centre in southern Santiago for periodic medical controls. Of these, 50 were bottle-fed (group A_1) and the remaining 15 were breast-fed at the time the study was carried out (group A_2). As is usual in Chile in this socioeconomic group, they also received fruit juices, puréed fruits and herb teas.

Group B included 100 school-age children, 8–10 years of age, from an elementary school in southern Santiago.

Group C was formed by 100 adults between 21 and 50 years of age, who were food handlers at a prepared-meal plant. This latter group was selected because as these individuals handle foodstuffs for rather considerable numbers of subjects (20000 rations per day) they may constitute a source of food-borne disease.

The number of individuals in each group was chosen to facilitate comparison between the groups, and was not representative of the distribution in the general population.

All techniques used to obtain the samples were carefully explained to the subjects or to their parents or guardians. A written consent form was signed for each specimen. The project was approved by the Ethics Committee of the Institute. Because these were healthy subjects, the Committee approved only one rectal swab in each subject. All samples were taken during March, April and May 1982.

Procedures. Faecal swabs were taken using Stuart's transport medium (Culturette, Marion Scientific Co. Kansas City, Mo., U.S.A.) and were plated within three hours.

They were cultured on eosin–methylene blue (EMB) and xylose–lysine–deoxycholate (XLD) agar plates. In addition, selenite F broth incubated at 35 °C for 14 h was used as enrichment medium for salmonellae, followed by subculturing in Salmonella–Shigella (SS) agar plates. The selective plates were incubated aerobically at 35 °C for 18–24 h. All culture media were purchased from Difco Laboratories, Inc. Colonies were identified by means of biochemical reactions according to Lennette *et al.* (1980). Serologic confirmation of enteropathogenic *Escherichia coli* 'classic serotypes' (EPEC), salmonellae and shigellae were carried out using poly- and monovalent antisera also from Difco. In addition, monovalent antiserum against *E. coli* 0142:K86 (Rowe & Gross, 1971), from Bio-Merieux (Marcyl-l'Etoile 69260 Charbonniéres-les Bains, France) was used. All monovalent antisera (0:K) were tested against live strains and bacteria heated at 100 °C for 60 min. Titres for EPEC were measured by serial dilution of the isolates. Those titres equal to or exceeding 1:320 for the homologous antiserum were considered positive. Samples from individuals of group B were investigated in addition for *Campylobacter jejuni* and *Yersinia enterocolitica*.

C. jejuni was isolated using the selective medium designed by Skirrow (1977), which consists of a blood agar (7% defibrinated horse blood) supplemented with antibiotics (Campylobacter Selective Supplement, Code SR-83, Oxoid Laboratories) and incubated at 42 °C for 48 h in a microaerophilic atmosphere containing 84% of N_2, 10% CO_2 and 6% O_2.

Colonies were selected by their appearance to the naked eye and their microscopic characteristics. Positive identification was achieved by techniques used routinely in our laboratory (Figueroa *et al.* 1980–1). Hippurate hydrolysis tests were used for differentiation of *C. jejuni* and *C. coli* isolates.

Y. enterocolitica was detected by the cold enrichment technique in phosphate buffer (Lennette *et al.* 1980). Subcultures were made on days 7, 15 and 30 in MacConkey agar. In addition, the EMB, XLD and SS plates used to isolate common pathogens were further incubated at 22 °C for 48 h after finishing the first stage of the study. Isolation was confirmed by biochemical tests as described by Lennette *et al.* (1980).

Enteropathogens isolated were tested for their sensitivity to five different antibiotics by the agar diffusion technique of Bauer *et al.* (1966). These were ampicillin, kanamycin, gentamicin, cotrimoxazole and furazolidone. An extended susceptibility test was used for multi-resistant strains using in addition streptomycin, chloramphenicol, tetracycline, carbenicillin, colistin sulphate, cephaloridine and nalidixic acid.

All strains that were resistant to at least three antibiotics were tested for their in vitro transfer capacity by conjugation using *E. coli* K 12 F^-, nalidixic acid-resistant (Curtiss, 1981). Exconjugants were detected using chloramphenicol (25 μg/ml) as counterselecting antibiotic. Testing was carried out at 37 °C. Those strains which did not transfer their resistance at this temperature were also tested at 22 °C.

Calculation of transfer was made applying the formula

$$\% \text{ transference} = \frac{\text{numbers of recombinant R colonies}}{\text{number of parental colonies}} \times 100.$$

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Table 1. *Frequency of isolation of bacterial enteropathogens from faeces of apparently healthy individuals* ($N = 265$). *Santiago, Chile*

Bacterial species	Group A: Bottle-fed group A_1 ($N = 50$)	Group A: Breast-fed group A_2 ($N = 15$)	Group B: school-age children ($N = 100$)	Group C: adults ($N = 100$)	Total ($N = 265$)
EPEC	10 (20%)	4 (20%)	4 (4%)	3 (3%)	21
Salmonellae	0	0	0	1 (1%)	1
Shigellae	0	0	2 (2%)	0	2
Campylobacter jejuni	ND	ND	1 (1%)	ND	
Yersinia enterocolitica	0	0	1 (1%)	0	1
Total recovery	10	4	8 (8%)	4	26
Total number of individuals	10	3*	8	3* (3%)	24

* One individual of group A_2 and one from group C harboured two enteropathogens.
ND = not done.

Strains in which transference was proved were assayed to rule out transformation as a genetic mechanism. For this purpose, filtrates through 0·45 μm Millipore membrane from the donor strain were tested with the same procedures used to study conjugation as described above.

RESULTS

Twenty-four of the 265 apparently healthy individuals included in this study harboured enteropathogens (9·1%). The total number of isolates was 26. The bacterial species isolated were EPEC in 21 instances, salmonella in one and shigella in two. *Y. entercolitica* and *C. jejuni* were investigated only in the school-age children. One of each of these organisms was isolated. The distribution of the isolates by age group is shown in Table 1. One subject harboured two strains of EPEC and another, one strain of EPEC and *Salmonella paratyphi B* (Table 1). Frequencies of isolation for all pathogens found in each of the age groups are shown in Table 2.

Susceptibility to antibiotics of strains from bottle-fed infants (group A_1) is shown in Table 3. In individuals from group A_2 (breast-fed) one strain of *E. coli* (0142:K86) was resistant to cotrimoxazole; in group B (school-age) one of the two isolates of *Shigella flexneri* 2 was resistant to ampicillin while one of the *E. coli* 026:K60 isolates proved to be resistant to kanamycin and cotrimoxazole. The only strain of *C. jejuni* detected was sensitive to all the above-mentioned antibiotics and in addition to erythromycin, tetracycline and colistin sulphate. As expected, this strain was resistant to cephalosporins. All strains from individuals from Group C were sensitive to the antibiotics tested.

Four of the strains isolated in group A_1 proved to be multi-resistant and were tested with seven additional antibiotics (Table 3). Two of these four strains were *E. coli* 0111:K58 and the remainder were *E. coli* 044:K74 and *E. coli* 020a020c:K61.

Table 2. *Bacterial species and serotypes isolated in 265 apparently healthy individuals, Santiago, Chile*

Bacterial species and serotypes	Group A_1 ($N = 50$)	Group A_2 ($N = 15$)	Group B ($N = 100$)	Group C ($N = 100$)
E. coli				
0111:K58	2	0	0	0
020a020c:K61	1	0	0	0
0127:K63	1	0	0	0
0128:K67	1	0	0	0
086:K61	1	1	0	0
026:K60	1	1*	2	0
0142:K86	0	1*	1	0
018a018c:K77	0	1	0	0
055:K59	0	0	1	0
0126:K71	0	0	0	1
0112a0112c:K66	0	0	0	1**
Shigella flexneri 2	0	0	2	0
Salmonella paratyphi B	0	0	0	1**
Campylobacter jejuni	ND	ND	1	ND
Yersinia enterocolitica	ND	ND	1	ND

*, ** Indicate that these isolates were obtained from one individual each.

Table 3. *Susceptibility to antibiotics of* E. coli *strains isolated from faeces of bottle-fed 3- to 6-month-old children (group A_1)*

	Antibiotic susceptibility of serotypes tested									
	(1)	(1)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(7)
Ampicillin	S	R	R	R	S	S	S	R	R	R
Kanamycin	S	S	S	R	S	S	S	S	R	R
Gentamicin	S	S	S	S	S	S	S	S	S	S
Cotrimoxazole	S	R	R	S	S	S	S	S	S	S
Furazolidone	S	S	S	S	S	S	S	S	S	S
Streptomycin	—	R	—	R	—	—	—	—	R	R
Chloramphenicol	—	R	—	R	—	—	—	—	R	R
Tetracycline	—	R	—	R	—	—	—	—	S	S
Carbenicillin	—	R	—	R	—	—	—	—	R	R
Colistin sulphate	—	R	—	S	—	—	—	—	S	S
Cephaloridin	—	R	—	S	—	—	—	—	R	R
Nalidixic acid	—	S	—	S	—	—	—	—	S	S

(1) 044:K74; (2) 020ac:K61; (3) 026:K60; (4) 0128:K67; (5) 086:K61; (6) 0127:K63; (7) 0111:K58; S, susceptible; R, resistant.

Under the conditions used in this study, neither *E. coli* 044:K74 nor *E. coli* 020a020c:K61 were able to transfer by conjugation their pattern of antibiotic resistance. On the other hand, both strains of *E. coli* 0111:K58 had transferred some of their antibiotic resistance traits to the *E. coli* receptor strain. The first *E. coli* 0111:K58 strain transferred genetic information for resistance to chloramphenicol, kanamycin and streptomycin with a frequency of 8×10^{-5}; the second strain of this bacterium transferred resistance to chloramphenicol and kanamycin with a frequency of $3{\cdot}1 \times 10^{-7}$. Transfer of the Lac+ phenotypic trait was also detected in the last strain. Transformation was not demonstrated for any of the four multi-resistant strains.

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DISCUSSION

Studies carried out during outbreaks of acute diarrhoea in newborn nurseries during the late forties and early fifties demonstrated that EPEC was the associated agent in many instances. The use of serological methods to identify these bacteria demonstrated that there was a variety of serotypes that could be isolated during these episodes. The discovery that strains of *E. coli* sometimes secrete toxins that induce diarrhoea in animals and in humans cast doubts about the causative role of EPEC in acute diarrhoea. However, studies in volunteers (Levine *et al.* 1978) demonstrated that the 'classic serotypes' do indeed cause diarrhoea by mechanisms that do not depend on the production of either the heat-labile or heat-stable toxins. In fine-structural studies of the intestinal epithelium of subjects with diarrhoea associated with EPEC a close attachment between the bacteria and the absorptive cells has been demonstrated, with extensive damage to the brush border (Ulshen & Rollo, 1980), Rothbaum *et al.* 1982). Taking into account the various mechanisms, including invasiveness, through which *E. coli* may cause its deleterious effects, the enteropathogenic, classic serotypes remain the most frequent agents of acute diarrhoea in many underdeveloped countries (Toledo *et al.* 1983).

Of three groups studied, the highest incidence of the carrier state was found among infants. Of those who were breast-fed, 20 % excreted EPEC. Unfortunately, the number of individuals studied in this group was small. Among the bottle-fed infants this proportion was similar to the previous groups (also 20 %). These high figures may be explained by the fact that the fruit purées, juices and infusions offered to infants in Chile since about three months of age, irrespective of whether or not they are breast-fed, become contaminated during preparation and/or storage (Araya *et al.* 1982).

Studies carried out by us and by other groups in underdeveloped countries show that maternal milk protects against the appearance of diarrhoeal episodes (Gordon, 1971; Gothefors *et al.* 1976; Gurwith *et al.* 1978; Hanson & Winberg, 1972). The fact that in this study both groups have comparable carriage rates for enteropathogens suggests that while breast milk protects against episodes of diarrhoea, it does not hinder the transit of pathogenic bacteria along the gastrointestinal tract (Gothefors *et al.* 1976). Thus, in the absence of diarrhoea it is still possible to detect the pathogens in the faeces of a high proportion of breast-fed infants. Rowland *et al.* (1980) in the Gambia found that 42·6 % of asymptomatic, breast-fed infants excreted EPEC. By contrast, Gurwith *et al.* (1978) in Canada found that less than 1 % of breast-fed infants, who were free from any gastrointestinal symptoms, excreted EPEC in the faeces. The level of carriage found by us in this age group in Chile appears to be intermediate between those reported in the above-mentioned studies. Excretion of EPEC by breast-fed, asymptomatic infants may be considered another manifestation of microbial contamination of the environment.

Recovery of enteropathogens decreased to 8 % among the school-age children and to 3 % among the adults. This reduction may be the result of the appearance of local immunity due to repeated contact with these agents.

EPEC were the most frequent isolates, and the serotypes found in this study are comparable to those reported in previous studies in Santiago (Figueroa, 1981).

There are very few publications concerning isolation of *C. jejuni* and *Y. entero-*

colitica in acute diarrhoea in Chile (García *et al.* 1981). Information about the carrier state for these bacteria in healthy individuals is scarce. This makes it difficult to interpret our results in school-age children. Bokkenheuser *et al.* (1979) has stated that in South Africa almost 40% of apparently healthy Black infants excrete *C. jejuni*. Unfortunately we could not study these agents among infants and adults and we feel that the levels of carriage found in school-age children cannot be considered representative of what happens in other age groups.

Most of the enteropathogens isolated were sensitive to the majority of the antibiotics tested. Thus 73·1% were susceptible *in vitro* to ampicillin, 85% to kanamycin, chloramphenicol and cotrimoxazole. All strains were sensitive to furazolidone and gentamycin.

Multi-resistant bacteria were isolated only from bottle-fed infants (28·6%). These results are in agreement with data published by Feeney, Cooke & Shinebaum (1980) for intestinal non-pathogenic bacteria. Our isolates were all EPEC and belonged to three serotypes. One explanation for this finding may be that artificially fed infants develop diarrhoea or other infectious diseases more often than breast-fed infants, and that for these episodes they are usually given antibiotics, by parental or non-medical prescription. This would select the patterns of resistance observed in the strains isolated here. Of the four strains of *E. coli* found to be multi-resistant in this study, two were *E. coli* 0111:K58. Coincidentally this is the serotype most frequently isolated from children hospitalized for acute diarrhoea in Chile (Figueroa, 1981). Both *E. coli* 0111:K58 strains transferred some of their antibiotic resistance traits by conjugation. This strongly supports the idea that these determinants are plasmid-mediated.

It has been suggested that the administration of low doses of antibiotics to malnourished children may result in significant improvements of their nutritional status, probably through 'favourable' modifications in their intestinal microecology (Luckey & Meier, 1972; Rosenberg *et al.* 1974). Our results point out some of the risks that may be associated with such procedures, and emphasize the need for caution in the use of antibiotics. This care should be exerted not only in administering these substances to humans but also to animals, from which antibiotic-resistant bacteria may originate (Levy, Fitzgerald & Macone, 1976).

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