Epidemiol. Infect. (1998), **121**, 587–598. Printed in the United Kingdom

High turnover rate of *Escherichia coli* strains in the intestinal flora of infants in Pakistan

I. ADLERBERTH[1]*, F. JALIL[2], B. CARLSSON[1], L. MELLANDER[3], L. Å. HANSON[1], P. LARSSON[4], K. KHALIL[5] AND A. E. WOLD[1]

[1] *Department of Clinical Immunology, University of Göteborg, Guldhedsgatan 10, S-413 46 Göteborg, Sweden*
[2] *Department of Social and Preventive Pediatrics, King Edward Medical College, Lahore, Pakistan*
[3] *Department of Paediatrics, University of Göteborg, Sweden*
[4] *Department of Clinical Bacteriology, University of Göteborg, Sweden*
[5] *Department of Paediatrics, King Edward Medical College, Lahore, Pakistan*

(*Accepted 18 July 1998*)

SUMMARY

The *Escherichia coli* flora of infants in developed countries is dominated by one or a few strains which persist for prolonged periods of time, but no longitudinal studies have been performed in developing countries. To this end, we studied the rectal enterobacterial flora in 22 home-delivered Pakistani infants during their first 6 months of life. Three colonies were isolated and species typed on each of 11 sampling occasions. *E. coli* isolates were strain typed using electromorphic typing of cytoplasmic enzymes, and their O serogroups were determined. There was a very rapid turnover of enterobacterial strains in the rectal flora of individual infants. On average, 8·5 different *E. coli* strains were found per infant, and several biotypes of other enterobacteria. Less than 50% of the infants were colonized with *E. coli* from their mothers, but strains of maternal origin were four times more likely to persists in the infants' flora than other *E. coli* strains. Enterobacteria other than *E. coli* were always of non-maternal origin, and *Enterobacter cloacae* and *Klebsiella pneumoniae* biotypes recovered from contaminated feeds were later identified in the infants' rectal flora. An early colonization with klebsiella or enterobacter was significantly associated with diarrhoea during the neonatal period, although these bacteria were not likely to be the cause of the disease. The results suggest that poor hygienic conditions result in an unstable and diverse enterobacterial flora, which may influence infant health.

INTRODUCTION

Escherichia coli and other enterobacteria are among the first colonizers of the newborn infant's intestine [1]. These bacteria may be acquired from the mother's faecal microflora during delivery [2], or be transferred between infants via the nursing staff [3, 4]. Another possible route of exposure is ingestion of contaminated feeds. In many traditional societies, even newborn infants considered to be 'fully breastfed' receive a number of different remedies in addition to breast milk [5]. It is likely that these foods are bacterially contaminated.

There is a constant turnover of *E. coli* strains in the microflora of an individual. Some *E. coli* strains are resident in the normal flora. A resident strain is usually defined by its presence for several weeks in succession. Transient strains, on the other hand, do not colonize. They may be found in the microflora on a single occasion, or on a few occasions closely spaced in time [6]. Newborn infants in developed countries as a role acquire a resident *E. coli* strain early after delivery and keep it in their intestinal microflora for

* Author for correspondence.

many months [7, 8]. Few additional *E. coli* strains are isolated from an infant harbouring a resident *E. coli* strain [8]. In developing countries, the environmental exposure to bacteria is likely to be pronounced, which could affect the intestinal microflora. For example, Pakistani newborn infants delivered in a hospital carry a larger number of enterobacterial species in their intestinal microflora than Swedish infants. This includes infants delivered by caesarean section who have not been in contact with maternal rectal bacteria [9]. Other studies have shown a more rapid acquisition of enterococci and lactobacilli in infants in developing countries, as compared to Western societies [10]. Whether this difference in exposure will result in an increased turnover of individual bacterial strains in the microflora has not been studied, since no longitudinal studies including typing of specific strains have been performed in developing countries.

The aim of the present study was to follow the enterobacterial colonization pattern in Pakistani home-delivered infants during the first 6 months of life. Individual *E. coli* strains were typed on the basis of their isoenzyme patterns, and grouped into resident and transient strains. The maternal rectal flora was sampled at delivery and infant feeds were cultured for the presence of enterobacteria to determine the origin of enterobacteria colonizing the newborn infant.

METHODS

Subjects and sampling

Twenty-two infants (10 girls and 12 boys) born in an urban slum in Lahore, Pakistan, were included in the longitudinal study of the rectal flora. The infants were delivered in their homes with the assistance of traditional birth attendants. From 16 out of the 22 mothers a sample of rectal flora was obtained during delivery. The families were visited by a trained health worker within 2 h after delivery, on three occasions during the first week (most often on days 2, 4 and 7), weekly during the first month and monthly thereafter. On each visit, the mother was questioned about the baby's feeding habits and signs of infection, a clinical examination of the child was performed and the child's rectal flora was sampled. Four infants dropped out of the study due to family migration (3 at 1 month of age and 1 at 5 months of age).

A second group of six home-delivered Pakistani neonates in the same area were visited on a few occasions during the first week after birth (1–5 occasions). The infant's rectal flora was sampled, and aliquots of the different fluids and foods given to the baby on that day were collected in sterile containers for determination of enterobacterial content.

Informed consent from the parents was obtained and the study was approved by the ethics committees of the Pakistani Medical Research Council and of Göteborg University, Sweden.

Bacterial cultivation and species identification

The rectal swab was rolled over a Drigalski agar plate [11] at bedside. In the local laboratory, the inoculate was spread with a sterile platinum loop and the plate was incubated aerobically at 37 °C overnight. The three last free-lying colonies were picked from the plate. This procedure gives a 97% probability of including the dominant strain, i.e. the strain most common when examining 10 randomly selected colonies from a rectal sample [12]. The food samples were cultured on Drigalski agar and colonies of different morphology were picked for analysis.

After subculture to check for purity, species identification was performed by biotyping [13]. This also permitted identification of different *E. coli* or other enterobacterial biotypes. Isolates were tested for mucoid growth, motility, indole production, urea, citrate, malonate, ornitine-decarboxylase, lysine-decarboxylase, lactose, sucrose, dulcitol, L-rhamnose, L-sorbose and esculine. For some isolates, the panel of tests also included *O*-nitrophenyl-D-galactopyranoside (ONPG), arabinose, H_2S and oxidase, or API 20E strips were used (API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France).

E. coli clonal analysis by electromorphic typing

The clonal identity of *E. coli* isolates was determined using electromorphic typing of eight different chromosomally encoded enzymes [14]. From each sampling occasion, all *E. coli* isolates with a unique biotype were analysed, as well as at least two of those isolates which shared a specific biotype.

The method was performed as described by Caugant, but with the use of polyacrylamide gel electrophoresis and blotting instead of starch gel electrophoresis [14, 15]. One loopful of a fresh bacterial culture was inoculated in 100 ml of nutrient broth (8 g/l; Difco, Detroit), and grown overnight with shaking at 37 °C. Bacteria were spun down at

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

37000 ***g*** for 20 min, resuspended in 2 ml buffer (0·01 M Tris, 0·01 M EDTA, pH 6·8), and sonicated with cooling for 1 min. Cell debris was spun down (37000 ***g***, 4 °C, 20 min) and the supernatant, containing bacterial enzymes, was stored at −70 °C until analysed.

Electrophoresis was performed in vertical polyacrylamide slab gels (7·5% polyacrylamide with 2·6% bisacrylamide for cross linking), using a 75 mM Tris buffer with 48 mM boric acid, pH 8·9, both in the gel and as running buffer. Twenty-five μl of bacterial supernatant in sample buffer (20% glycerol, 0·5% (w/v) bromic phenol blue) was loaded in each gel pocket, and the electrophoresis was run at 15 mA. Up to four copies of the separation were made by a series of electrophoretic transfers onto DEAE paper (DE81, Whatman International Ltd, Maidstone, England) using 7·5 mM Tris buffer with 4·8 mM boric acid, pH 8·9, as transfer buffer. Each DEAE paper was stained with the appropriate enzyme substrate and chromophores [14] for the following eight enzymes: malate dehydrogenase, 6-phosphogluconate dehydrogenase, adenylate kinase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, mannose phosphate isomerase, phenylalanyl-leucine peptidase and leucyl-glycyl-glycine peptidase. The migration of each enzyme was compared with two standards run in parallel.

E. coli isolates from a single child were assayed together; isolates that shared electrophoretic mobility for each of the eight enzymes tested were considered as belonging to one strain [14]. No comparisons were done between infants.

Serotyping

One isolate from each *E. coli* strain was serotyped using antisera to 69 common *E. coli* O antigens [16].

Characterization of enterobacteria recovered in connection to diarrhoeal episodes

Each *E. coli* strain and each biotype of *Klebsiella*, *Enterobacter* or *Citrobacter* spp. isolated from an infant the week before or during a diarrhoeal episode were tested for enterotoxin production by ELISA and adhesin expression by haemagglutination (HA).

For HA, the isolates were tested after overnight culture on tryptic soy agar plates, and after three passages in static Luria broth [17, 18]. Twenty-five μl of a thick bacterial suspension in PBS ($> 10^{10}$ bacteria/ml) was mixed on a microscope slide with 25 μl of a 3% suspension of human, chicken or guinea-pig erythrocytes in PBS containing 2·5% methyl-α-D-mannoside (to inhibit HA caused by mannose-specific type 1 fimbriae) [17]. Agglutination was read by the naked eye after gentle tilting of the slide for 3 min, and designated as positive or negative. The agglutination of human, chicken or guinea-pig erythrocytes in presence of methyl-α-D-mannoside was designated as mannose-resistant (MRHA).

Assessment of the production of heat labile (LT) and heat stable (ST) enterotoxin was performed on three individual colonies from each isolate by means of ganglioside GM1-ELISAs [19, 20], using the direct culture in plate method [21]. Bacteria from an overnight blood agar culture were inoculated in Luria broth containing 45 μg lincomycin and 2·5 mg glucose per ml and added to GM1-coated and bovine serum albumin-blocked microtitre plates. The plates were incubated with shaking at 37 °C overnight and the supernatants were saved for ST determination. The plates were washed and developed for GM1-bound LT by adding a mouse monoclonal antibody against LT, followed by anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) and enzyme substrate [20]. The presence of ST type a in the supernatant was determined with a GM1-ELISA inhibition method, in which the ability of the supernatants to block the specific binding of a STa-specific monoclonal antibody to solid-phase-bound ST ganglioside (GM1-bound ST-cholera B subunit) was tested [20, 21].

Statistics

Fisher's exact test and Mann–Whitney *U* test were used for significance testing.

RESULTS

Infant health

Six of the 22 infants (27%) experienced a diarrhoeal episode during the study period, all within the first month. The occurrence of diarrhoea peaked at 1 week of age when four infants were ill.

Six infants had an episode of respiratory tract infection, which occurred between 2 weeks and 5 months of age, with a peak incidence at 3 months when four infants were ill.

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

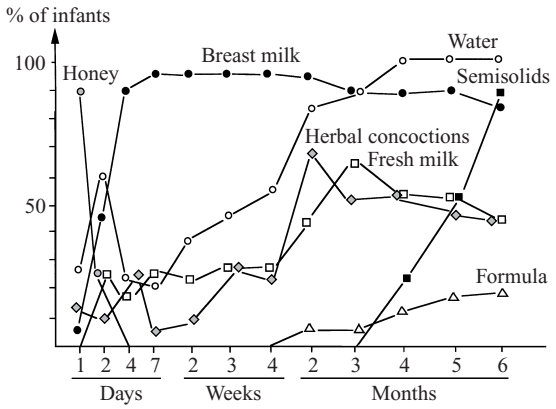

Fig. 1. Percentage of infants having received different fluids and food at various points in time after birth. Data from between 17 and 22 infants at each time point.

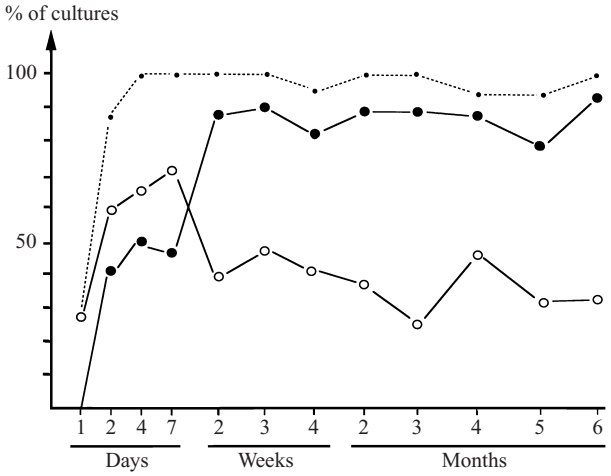

Fig. 2. Percentage of rectal cultures from Pakistani infants positive for Gram-negative bacteria (dotted line), *E. coli* (filled circles) and Gram-negative bacteria other than *E. coli* (open circles) at various times after birth. Between 17 and 22 infants contribute the data for each time point.

Feeding pattern

The feeding pattern of the 22 infants in the longitudinal study was recorded. All infants but one received breast milk, but breastfeeding was often initiated as late as 3 or 4 days after delivery and no infant was exclusively breastfed (Fig. 1). All infants received honey before the initiation of breastfeeding. Water or fresh animal milk (cow or buffalo) were commonly given in addition to breast milk, whereas few infants received commercial formula (Fig. 1). Traditional herbal concoctions were frequently given. Examples include ghutty and arq, which are used as laxatives, and gripe water, which is a remedy against infant colic. Semisolids, prepared from, for example, rice, potatoes or bananas, were introduced from the fourth month of age.

Enterobacterial colonization pattern

Twenty out of the 22 infants (90%) were colonized with Gram-negative bacteria in their intestines by days 2–3 and all infants were colonised by days 4–5 (Fig. 2). During the first week, enterobacteria other than *E. coli* dominated the flora (Fig. 2). Especially *Klebsiella pneumoniae* and *Enterobacter cloacae* were common during this period, and continued to be present in approximately one third of the infants throughout the 6 months of study (Table 1). *E. coli* has been present in the rectal flora of 73% of the infants 1 week old, in 95% of infants 1 month old, and in all infants at 2 months of age.

Two or more enterobacterial species were frequently isolated from one sample (Table 1).

Persistence of individual enterobacterial strains in the rectal flora

Individual strains of *E. coli* were identified by electromorphic typing of chromosomally encoded bacterial enzymes. The colonization patterns of the individual infants are shown in Figure 3.

As a mean, each infant carried on average 8·5 different *E. coli* strains during the first 6 months of life (the three infants followed for only 1 month not included). During this period, each infant contributed on average 11 samples. (Twelve sampling occasions were scheduled per infant. When a sample was not obtained as scheduled, this was because the mother and infant were not at home when the visit was paid.)

E. coli strains isolated on at least two occasions from samples at least 3 weeks apart were considered as resident strains. Strains found only once, or on a few occasions during a period shorter than 3 weeks, were considered as transient strains. If a scheduled monthly sampling was missed, strains found only on the occasion immediately before or after this occasion could not be defined as either transient or resident. The same was true for strains occurring at the last sampling occasion only.

Sixty-three percent of the infants (12/19) carried at least one resident *E. coli* strain during the 6 months period. However, only one infant (no. 16) carried a resident strain from her first to her sixth month of life (Fig. 3). Thus, resident strains were often replaced by

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

Table 1. *Frequencies of different aerobic Gram-negative species in rectal cultures from Pakistani infants and mothers*

	Frequency of isolation (% of cultures)						
	Infants						
Days after delivery…	1–3	4–7	14–21	30–60	90–120	150–180	Mothers
E. coli	19	49	90	85	89	86	75
K. pneumoniae	8·1	30	18	29	11	22	0
K. oxytoca	8·1	8·1	2·6	0	2·8	0	6·2
Other *Klebsiella* spp.	0	0	5·1	2·4	2·8	0	0
E. cloacae	11	22	13	4·9	14	11	6·2
E. aerogenes	0	2·7	2·6	0	0	0	0
Other *Enterobacter* spp.	11	5·4	2·6	0	5·6	0	12
C. freundii	0	5·4	5·1	2·4	0	5·5	6·2
Other *Citrobacter* spp.	2·7	11	0	0	2·8	0	6·2
Proteus spp.*	2·7	0	2·6	2·4	5·6	5·4	6·2
Serratia spp.	0	2·7	0	0	0	0	0
Other Gram-negatives†	13	5·4	5·1	2·4	8·4	8·1	6·2
> 1 species	16	32	33	29	36	27	19
> 2 species	5·4	8·1	10	2·4	8·3	11	6·2

Samples from the infants were obtained at delivery, on three occasions during the first week, weekly during the first months and monthly thereafter. Samples of the mothers' rectal flora were obtained at parturition. From each culture, three colonies obtained from a Drigalski agar plate were species typed. Twenty-two infants were sampled; 36–41 cultures were examined at each point in time indicated in the table. The maternal samples derived from 16 mothers.

* Including *Proteus* spp., *Providencia* spp. and *Morganella morganii*.

† Including *Pseudomonas*, *Acinetobacter* and not identified Gram-negative species.

other resident strains, or even lost and replaced by a succession of transient strains in the individual infant. On average, 1·2 different resident strains (range 0–5) were found in each infant, whereas a mean of 6·4 transient strains were identified per infant (range 1–13).

Nineteen percent (11/59) of the *E. coli* strains acquired during the infants' first month of life established as residents, compared with 14% (12/88) of the strains acquired at 2–5 months of age. The mean time of persistence of resident strains acquired during the first month of life was 76 days (range 23–170 days). The mean time of persistence of resident strains first appearing between 2 and 5 months was at least 60 days (75% of these strains were still present on the last sampling occasion, so no exact figure can be calculated).

Individual strains of other enterobacterial species than *E. coli* were defined according to their biotype (Fig. 3). In many instances, several different enterobacterial biotypes were present in a rectal sample, and several biotypes of *Klebsiella* or *Enterobacter* sp. could colonise an infant in succession. Certain enterobacterial biotypes seemed, however, to persist in an infant's flora for some time. Thus, six infants harboured *K. pneumoniae* of a particular biotype for more than 3 weeks in succession. Two infants harboured a specific *E. cloacae* biotype for more than 3 weeks (Fig. 3). *Citrobacter* species were only found as transient colonizers.

O serotype distribution in *E. coli* strains isolated from the rectal flora

The O serotypes of the *E. coli* strains are shown in Table 2. The O antigens most commonly encountered were O17/O77, O1 and O20. The serotypes O8, O16, O21 and O75 were also relatively frequent. However, as many as 50% of all strains were non-typable with the 69 O antisera used. Twelve percent of the strains were 'rough', i.e. agglutinated spontaneously in saline.

Only 16% of the strains belonged to any of the ten 'uropathogenic' serotypes O1, O2, O4, O6, O7, O8, O16, O18, O25 or O75, which are common in the intestinal microflora of Western individuals [22–24].

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

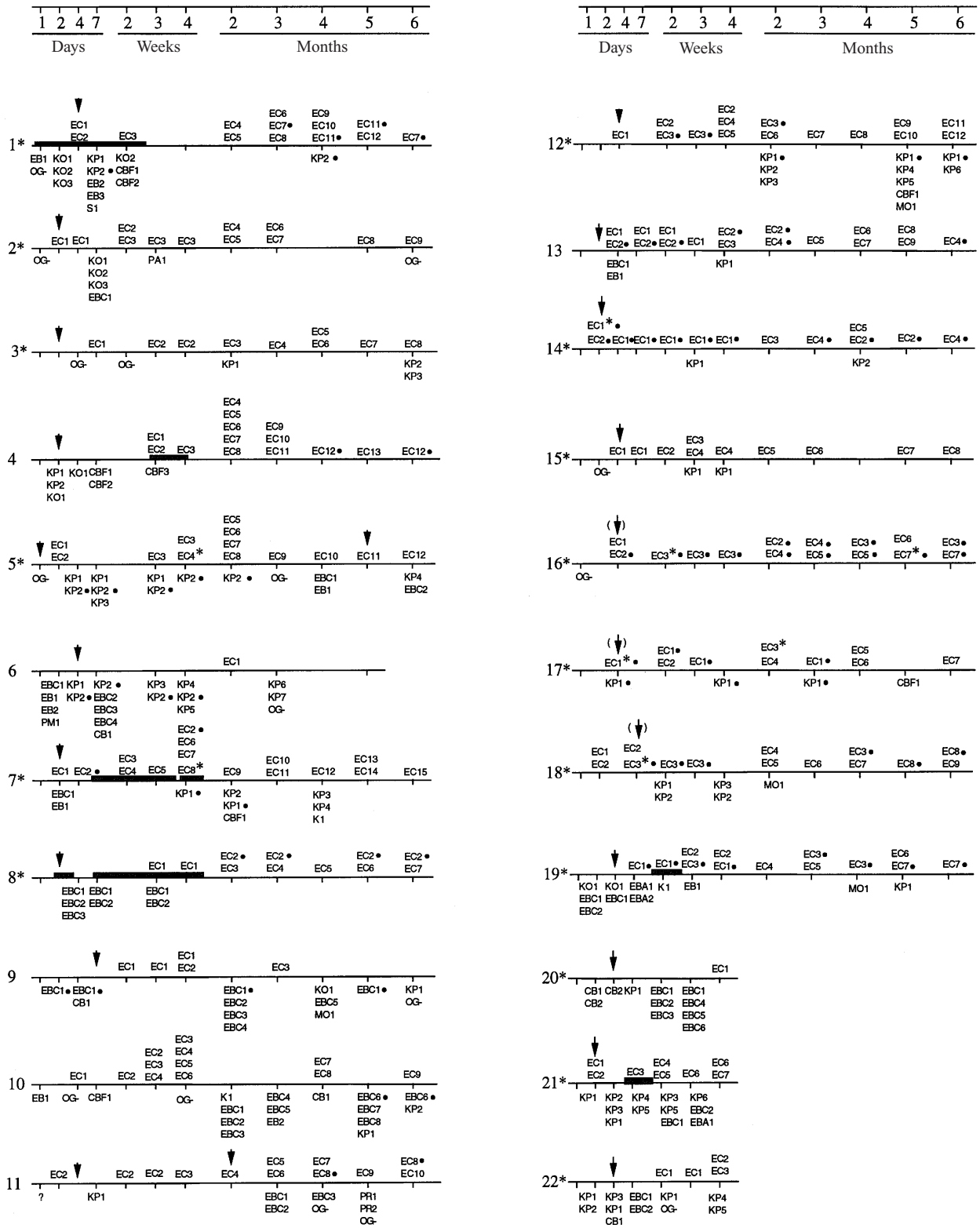

Fig. 3. Individual colonization patterns for each of the 22 infants followed longitudinally. Vertical lines denote sampling occasions. Arrows indicate time points for initiation and termination of breast-feeding (arrows in parentheses indicate that breast milk might have been given earlier). Diarrhoeal episodes are indicated with bars along the time axis. *E. coli* strains (EC) (above time axis) were identified by electromorphic typing and numbered according to their order of appearance in the infant's rectal flora. Resident *E. coli* strains are indicated by closed circles. Infants whose mothers' rectal samples were analysed are indicated by an asterisk (*), and *E. coli* strains of presumed maternal origin in these infants are similarly

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

Table 2. *O serotypes of rectal* E. coli *strains isolated from Pakistani infants 0–6 months of age*

O antigens	Number of strains (% of strains)
O1	7
O2	1
O3	1
O4	1
O6	2
O7	1
O8	4
O11	1
O12	1
O13	2
O15	3
O16	4
O17	5
O17/O77	2
O20	7
O21	4
O22	1
O25	2
O33	1
O42	1
O75	4
O77	1
O81	1
O85/O117	2
O86	2
O117	1
Typable	63 (38)
'Uropathogenic'	27 (16)
Non-typable	83 (50)
Rough	20 (12)
Total	166*

O serotyping was performed using antisera to 69 O antigens common among Swedish clinical isolates of *E. coli*. Non-typable signifies that the isolates were not agglutinated by any of these antisera, and 'rough' that they agglutinated spontaneously in saline. 'Uropathogenic' denotes strains belonging to either of the serotypes O1, O2, O4, O6, O7, O8, O16, O18, O25 or O75.

* Seven strains were not typed.

Maternal rectal flora as a source of enterobacteria

Cultures of the rectal flora were obtained from 16 mothers at delivery. Twelve of them harboured *E. coli*, and they contributed 24 different strains in total. Six infants (38%) became colonized with an *E. coli* strain present in his/her mother's flora at delivery (child nos. 5, 7, 14, 16, 17 and 18, Fig. 3), two of them with two different maternal strains each (nos. 16 and 17). The serotypes of the maternal strains transferred to the infants were O1, O75 and rough in one case each, while five strains were non-typable with the O antisera used. The maternal *E. coli* strains appeared in the rectal flora of the infants when the child was, on average, 3 weeks old (range 2–153 days).

Five out of the eight strains acquired from the mothers became resident in the infants. This included the O75 strain, the rough strain and three non-typable strains. Thus, *E. coli* strains acquired from the mother had a 62% chance of becoming resident in the infant's flora, as compared with 14% for other *E. coli* strains ($P = 0{\cdot}0046$, Fisher's exact test).

The mothers also harboured other enterobacterial species than *E. coli* in the rectal flora (Table 1), but these seemed never to be transferred to the infants. Thus, enterobacteria other than *E. coli* in the child's rectal flora were never of the same biotype as those found in the maternal rectal flora at delivery.

Infant feeds as a source of enterobacteria

A small study was performed to investigate if infant feeds could be a source of Gram-negative bacteria colonizing the infant during the first week of life. To this end, six infants were visited on a few occasions during their first week of life. Samples were taken from their feeds as well as from their rectal flora. The overall enterobacterial colonization pattern of these infants was similar to that of the first group of infants (Table 3).

Nineteen out of the 26 food samples obtained were contaminated with Gram-negative bacteria. Ten of the samples yielded *Enterobacteriaceae*, most frequently *E. cloacae* or *Klebsiella* spp., but *E. coli* was never found in a food sample (Table 3). Five out of the six infants received some feed contaminated with enterobacteria during their first days of life.

Two infants received feeds contaminated with *K. pneumoniae*. In one of these infants, a colonization with food strains suggestedly occurred. Two *K. pneumoniae* biotypes were found in the fresh animal

indicated by asterisks (*). Enterobacteria other than *E. coli* were identified by biotyping (below time axis) and numbered consecutively in order of appearance. Abbreviations: *K. pneumoniae* (KP), *K. oxytoca* (KO), other *Klebsiella* spp. (K), *E. cloacae* (EBC), *E. aerogenes* (EBA), other *Enterobacter* spp. (EB), *Citrobacter freundii* (CBF), other *Citrobacter* spp. (CB), *Serratia* spp. (S), *Proteus mirabilis* (PM), other *Proteus* spp. (PR), Providencia spp. (PA), *Morganella morganii* (MO) and other Gram-negatives (OG−). Persisting biotypes of *K. pneumoniae* and *E. cloacae* are indicated by filled circles.

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

Table 3. *Presence of various enterobacterial species in foods and fluids administered to Pakistani neonates, and in rectal cultures obtained from these neonates*

	Occurrence in food (% of samples)				Occurrence in infants' rectal cultures (% of cultures) ($n = 17$)
	Fresh* milk ($n = 12$)	Water ($n = 3$)	Boiled water ($n = 6$)	Honey† ($n = 5$)	
Gram-negatives	100	100	33	40	100
Enterobacteriaceae	50	33	33	20	82
E. coli	0	0	0	0	53
K. pneumoniae	25	33	0	0	41
K. oxytoca	8·3	0	0	0	12
E. cloacae	50	0	33	0	18
E. agglomerans	8·3	0	17	0	5·9
C. freundii	0	0	0	20	18
Other *Citrobacter* spp.	8·3	0	0	0	0
Serratia spp.	8·3	0	0	0	0
Other Gram-negatives‡	75	67	33	20	18

Different fluids and foods administered to six Pakistani neonates, and rectal samples obtained from these neonates during the first 5 days of life were cultured and examined for the presence of various enterobacterial species.
* Fresh cow or buffalo milk, undiluted or diluted in boiled water or tea.
† Pure honey, or honey diluted in boiled water.
‡ Including *Pseudomonas aeruginosa*, other *Pseudomonas*, *Alcaligenes* and not identified Gram-negative species.

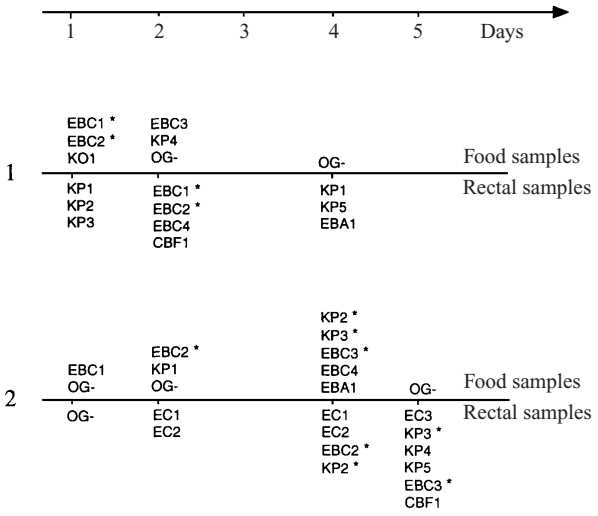

Fig. 4. Possible acquisition of intestinal enterobacteria from contaminated feeds. Enterobacterial colonization pattern of two infants and enterobacteria recovered from feeds administered to these infants during their first days of life. Vertical lines denote sampling occasions. In the individual infant, different biotypes of *E. coli* and other enterobacteria were numbered consecutively in order of appearance in the feeds or in the rectal flora. Specific biotypes identified in both feeds and rectal samples are indicated by an asterisk (*). Abbreviations: see Figure 3.

milk given to this infant on day 4. One of the biotypes appeared in the baby's intestinal flora on the same day, the other biotype one day later (Fig. 4). Three infants became colonized with *K. pneumoniae* from unidentified sources only.

Four infants received feeds contaminated with *E. cloacae*. In two of these infants, the same *E. cloacae* biotype was found in a feed and in the rectal flora 1 or 2 days later (Fig. 4). These two were the only infants in the group whose rectal samples yielded *E. cloacae*.

One food sample given to one infant yielded *C. freundii*, of a single biotype. This biotype appeared in the rectal flora of the infant on the day she received the contaminated food (data not shown). Two other infants became colonized with *C. freundii*, although this species was never detected in their feeds.

Diarrhoeal episodes in relation to enterobacterial colonization pattern

Six of the 22 infants followed longitudinally (27%) experienced an episode of diarrhoea during the study, all appearing during the first month of life (Fig. 3). We sought to determine whether any particular colonization pattern predisposed for diarrhoea. The microflora during the first week of life was therefore compared between those who later contracted diarrhoea and those who did not.

In infants contracting diarrhoea, 62% of the first

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

week's cultures contained *Klebsiella* or *Enterobacter* sp., as compared with 29% of the cultures from infants who remained healthy ($P = 0{\cdot}019$, Mann–Whitney U test). In contrast, the rate of isolation of *E. coli* during the first week of life was similar in infants contracting diarrhoea or staying healthy (28 *vs.* 35% of cultures positive for *E. coli*, respectively, $P = 0{\cdot}52$).

None of 18 *E. coli* strains isolated from infants the week before or during diarrhoea were positive for the production of heat labile enterotoxin (LT), whereas one strain, isolated from a child after 1 week of diarrhoea, was positive for the production of heat stable enterotoxin (ST). This strain also caused MRHA of human erythrocytes. The rest of the 18 *E. coli* strains were negative for MR adhesins. Two of these *E. coli* strains (11%) belonged to 1 out of 18 O serogroups characteristic of diarrhoeagenic *E. coli*: O6, O8, O15, O18, O20, O25, O26, O28, O78, O80, O85, O86, O111, O112, O115, O119, O125 and O128 [25], but this was also true for 13% of all *E. coli* strains. *Klebsiella*, *Enterobacter* and other non-*E. coli* enterobacteria isolated in connection to diarrhoea never expressed adhesins causing MRHA, nor did they produce ST or LT.

DISCUSSION

The intestinal enterobacterial colonization pattern was studied in Pakistani infants born in an urban slum area of Lahore and followed during their first 6 months of life. The infants were delivered at home with the aid of traditional birth attendants. Hygienic measures were limited to washing the mother's perineal area with warm water; no antiseptic agents were used. Still, only 38% of the infants acquired *E. coli* from their mothers, and other enterobacteria were never transferred from mother to infant. In fact, since some of the *E. coli* strains of suggested maternal origin appeared as dominant strains in the infant's intestinal microflora after several weeks or months, it is likely that they were transferred at a later time point. The vertical transfer rate of *E. coli* found in the Pakistani home-delivered infants is, thus, comparable to that previously reported from hospital births in industrialized societies, i.e. approximately 30% [2, 4, 22]. Low transmission rates have previously been ascribed to strict hygienic measures during delivery, including washing with antiseptics. Our data instead suggest that transfer of faecal bacteria is rare when the mother gives birth in a supine delivery position, and the baby is pulled out by an assistant. Kneeling or standing during delivery, as practised in, e.g. Guatemalan Indian villages [1], may increase the probability of faecal contamination of the baby and thereby transfer of bacteria from mother to infant.

Regardless of the low rate of transfer from the mother, practically all infants were colonized with enterobacteria within 2 days after birth. This figure is similar to the findings in previous studies of infants from developing countries [1, 9, 26], but is higher than reported from developed countries [9, 27, 28]. It is, thus, evident that the environment in Pakistan provides a rich source of enterobacteria capable of colonizing the infant's intestine. *Klebsiella* and *Enterobacter* spp., which dominated over *E. coli* during the first week, were likely to derive from contaminated feeds. Although all but one infant were breastfed, they also received a variety of feeds and remedies before breastfeeding was initiated and afterwards in addition to breast milk. In the Western world, *E. coli* usually dominates in healthy full-term neonates [27, 29], whereas *Klebsiella* and *Enterobacter* sp. dominate in neonatal intensive care units, which has been explained by a suppression of, i.e. *E. coli* by antibiotics [4, 30, 31]. Similar to the pattern in Western countries, the *Klebsiella* and *Enterobacter* strains were gradually replaced by *E. coli* in the intestinal microflora as the infants grew older, suggesting that the former are more sensitive to the suppressive effect of anaerobic bacteria which successively establish after birth [32].

During the 6 months we followed the infants, different strains of *E. coli* and other enterobacteria replaced one another in a rapid succession in the individual infant's intestinal flora. Only 1 out of 19 infants carried a resident strain during the whole 6 months period. This pattern is different from that previously recognized in infants in industrialized countries, who are often stably colonized for months with the first *E. coli* strain they acquire [7, 8]. Thus, 93% of Swedish infants colonized with *E. coli* at 6 weeks of age carry a resident *E. coli* strain, and these strains have a mean persistence time of 7 months [8]. Corresponding figures from our study were: 44% of infants colonized with *E. coli* at 1 month of age had acquired a resident strain, whose mean persistence time was 2·5 months. The Swedish infants were followed for between 11 and 18 months, during which period they acquired a mean of 4·2 different *E. coli* strains each, contrasting with the 8·5 strains per infant found here during a considerably shorter time (6 months). As the Swedish study obtained samples less frequently than ours, the total number of sampling

https://doi.org/10.1017/S0950268898001484 Published online by Cambridge University Press

occasions was similar, but they examined a larger number of colonies from each sample, which would facilitate the detection of additional *E. coli* strains [8].

Despite the overall low tendency of individual *E. coli* to persist in the Pakistani infants' intestinal flora, most strains transferred from the mother established as residents in the infant. Thus, non-maternal strains seemed poorly adapted to the human host. Some of them may, in fact, derive from animals, which are often kept in the immediate surroundings. In contrast, infants born in Western countries face a set of selected hospital enterobacterial strains transferred between infants at the maternity ward, and probably exclusively adapted to colonizing the intestinal tract of the newborn infant [8].

The O-serotype distribution among *E. coli* strains from Pakistani infants differed from that previously reported from industrialized societies. Only 16% of the strains belonged to any of ten recognized 'uropathogenic' O groups, i.e. O1, O2, O4, O6, O7, O8, O16, O18, O25 and O75, whereas approximately one third of *E. coli* strains isolated from the rectal flora of neonates [22], older children [23] or adults [24] in industrialized countries belong to these serotypes. Furthermore, 50% of the Pakistani strains were non-typable with the antisera used, in contrast to around 20% of strains from Swedish neonates [2] or older children [23]. Strains of maternal origin colonizing the Pakistani infants, i.e. strains which are likely to be well adapted to the human host, did not differ in serotype distribution from strains of non-maternal origin, indicating that predominant serogroups in the human faecal flora may actually differ between Pakistan and Sweden. To our knowledge, there are no previous similar studies of *E. coli* serotypes of individuals in developing countries.

Aerobic or facultative bacteria like *E. coli* elicit specific antibody responses when colonizing the gut [33]. The constant introduction of new bacterial strains and species into the intestinal microflora may provide a strong stimulus to the immune system. Accordingly, Pakistani infants show a strong salivary secretory IgA response against a pool of *E. coli* O antigens at 2 months of age, while a similar level is not reached by Swedish infants until 1–2 years of age [34].

One may ask whether the intestinal enterobacterial colonization pattern in the Pakistani infants has any consequences to health. Several studies indicate that neonatal septicaemia is caused by enterobacteria translocating from the gut [35, 36]. The constant exposure of the Pakistani infant to new enterobacterial strains would increase the probability of encountering virulent strains, and may also contribute to the very high frequency of neonatal septicaemia in this country [37]. *Klebsiella* sp. accounts for 30% of the cases of neonatal septicaemia in Pakistan [38], and the high colonization rate with this bacterium in very young Pakistani infants may be a cause of this dominance.

A colonization with *Klebsiella* or *Enterobacter* sp. during the first week increased the apparent risk of diarrhoea during the first weeks of life. Diarrhoea is known to induce disturbances in the normal intestinal microflora [39], but the early colonization with *Klebsiella* and *Enterobacter* sp. was probably not a result of the disease, since the occurrence of diarrhoea did not peak until 7 days of age. Neither do we believe that these bacteria were the direct cause of the disease. *Klebsiella* sp. may produce ST and LT [40–42], perhaps as a consequence of transfer of virulence plasmids from *E. coli* [43], but we could not detect production of ST or LT enterotoxins in the *Klebsiella* or *Enterobacter* strains found in association with a diarrhoeal episode. Thus, we favour the explanation that the frequent colonization with *Klebsiella* and *Enterobacter* sp. simply reflected that the intestinal microflora in these infants provided poor 'colonization resistance' against *Klebsiella* and *Enterobacter* sp. as well as against unidentified diarrhoeal pathogens, possibly due to a poorly developed anaerobic microflora [44–46].

In summary, the Pakistani newborn infants exhibit an unstable enterobacterial flora with a constant renewal of strains, a pattern which may have consequences for infant health. We believe the high strain turnover reflects a heavy environmental exposure to enterobacteria. Breastfeeding has been shown to reduce colonization with *Klebsiella*, *Enterobacter* and *Citrobacter* sp. [9, 47] and also to create a more stable and less diverse *E. coli* flora [47, 48]. In addition, exclusive breastfeeding would prevent exposure to enterobacteria present in contaminated feeds. Thus, in poorly developing countries, exclusive breastfeeding from the day of birth should be promoted.

ACKNOWLEDGEMENTS

We thank Eva Ågren, Maria Badenfors and Leif Larsson for skilful technical assistance, and Khadija Mazhar for excellent help in the Microbiology laboratory in Lahore. We thank Dr Ann-Mari Svennerholm at the Department of Medical Micro-

biology and Immunology, Göteborg University, for kindly providing reagents and antibodies for the GM1-ELISAs, and Gudrun Wiklund for technical advice. We also thank Dr Bertil Kaijser at the Clinical Bacteriology Department, Sahlgren's Hospital, for O-serotyping the *E. coli* strains. The study was supported by grants from SAREC (the Swedish Agency for Research Cooperation with Developing Countries), the Swedish Medical Research Council (grant no. 215), and the Medical Faculty of Göteborg University.

REFERENCES

1. Mata LJ, Urrutia JJ. Intestinal colonization of breastfed children in a rural area of low socio-economic level. Ann NY Acad Sci 1971; **176**: 93–109.
2. Bettelheim KA, Breadon A, Faiers MC, O'Farell SM, Shooter RA. The origin of O-serotypes of *Escherichia coli* in babies after normal delivery. J Hyg 1974; **72**: 67–70.
3. Bettelheim KA, Teoh-Chan C, Chandler M, et al. Spread of *Escherichia coli* colonizing newborn babies and their mothers. J Hyg 1974; **73**: 383–7.
4. Fryklund B, Tullus K, Berglund B, Burman LG. Importance of the environment and the faecal flora of infants, nursing staff and parents as sources of Gram-negative bacteria colonizing newborns in three neonatal wards. Infection 1992; **20**: 253–7.
5. Ashraf RN, Jalil F, Khan SR, et al. Early child health in Lahore, Pakistan: V. Feeding patterns. Acta Paediatr Suppl 1993; **390**: 47–61.
6. Sears HJ, Brownlee E, Uchiyama JK. Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. J Bacteriol 1949; **59**: 299–301.
7. Wiedemann B, Habermann R, Knothe H, Ihrig L. Untersuchungen über die stabilität der Koli-flora des gesunden menschen. 3. Mitteilung: Über dasvorkommen permanenter und passanter typen bei kleinkindern. Arch Hyg 1971; **154**: 581–9.
8. Kühn I, Tullus K, Möllby K. Colonization and persistence of *Escherichia coli* phenotypes in the intestines of children aged 0 to 18 months. Infection 1986; **14**: 7–12.
9. Adlerberth I, Carlsson B, de Man P, et al. Intestinal colonization with *Enterobacteriaceae* in Pakistani and Swedish hospital delivered infants. Acta Paediatr Scand 1991; **80**: 602–10.
10. Bennet R, Eriksson M, Tafari N, Nord C-E. Intestinal bacteria of newborn Ethiopian infants in relation to antibiotic treatment and colonization with potentially pathogenic Gram-negative bacteria. Scand J Infect Dis 1991; **23**: 63–9.
11. Kauffman F, ed. The bacteriology of *Enterobacteriaceae*, 2nd ed. Copenhagen: Munksgaard, 1969.
12. Lidin-Janson G, Kaijser B, Lincoln K, Olling S, Wedel H. The homogeneity of the faecal coliform flora of normal schoolgirls, characterized by serological and biochemical properties. Med Microbiol Immunol 1978; **164**: 247–53.
13. Bettelheim KA, Taylor JA. A study of *Escherichia coli* isolated from chronic urinary infection. J Med Microbiol 1969; **2**: 225–36.
14. Caugant DA. Enzyme polymorphism in *Escherichia coli*: genetic structure of intestinal populations, relationships with urinary tract infection strains and with *Shigella*. Göteborg, Sweden: Göteborg University, 1983.
15. McLellan T, Ramshaw JAM. Serial electrophoretic transfers: A technique for the identification of numerous enzymes from single polyacrylamide gels. Biochem Genetics 1981; **19**: 647–54.
16. Lidin-Janson G, Falsen E, Jodal U, Kaijser B, Lincoln K. Characteristics of antibiotic resistant *Escherichia coli* in the rectum of healthy schoolchildren. J Med Microbiol 1977; **10**: 299–308.
17. Duguid JP, Gillies RR. Fimbriae and adhesive properties in dysenteric bacilli. J Pathol Bacteriol 1957; **74**: 397–411.
18. Wold AE, Caugant DA, Lidin-Janson G, de Man P, Svanborg C. Resident colonic *Escherichia coli* strains frequently display uropathogenic characteristics. J Infect Dis 1992; **165**: 46–52.
19. Svennerholm A-M, Wiklund G. Rapid GM1-enzyme-linked immunosorbent assay with visual reading for identification of *Escherichia coli* heat-labile enterotoxin. J Clin Microbiol 1983; **17**: 596–600.
20. Svennerholm A-M, Wikström M, Lindblad M, Holmgren J. Monoclonal antibodies against *Escherichia coli* heat-stabile toxin (ST) and their use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay. J Clin Microbiol 1986; **24**: 585–90.
21. Svennerholm A-M, Lopez-Vidal Y, Holmgren J, McConnell MM, Rowe B. Role of PCF8775 antigen and its coli surface subcomponents for colonization, disease and prospective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect Immun 1988; **56**: 523–8.
22. Gothefors L, Carlsson B, Ahlstedt S, Hanson LÅ, Winberg J. Influence of maternal gut flora and colostral and cord serum antibodies on presence of *Escherichia coli* in faeces of the newborn infant. Acta Paediatr Scand 1976; **65**: 225–32.
23. Lidin-Janson G, Hanson LÅ, Kaijser B, et al. Comparison of *Escherichia coli* from bacteriuric patients with those from feces of healthy schoolchildren. J Infect Dis 1977; **136**: 346–53.
24. Siitonen A. *Escherichia coli* in fecal flora of healthy adults: Serotypes, P and Type 1C fimbriae, non-P mannose-resistant adhesins, and hemolytic activity. J Infect Dis 1992; **166**: 1058–65.
25. Levine MM. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J Infect Dis 1987; **155**: 377–89.
26. Rotimi VO, Olowe SA, Ahmed I. The development of

bacterial flora in premature neonates. J Hyg 1985; **94**: 309–18.
27. Tullus K. Fecal colonization with P-fimbriated *E. coli* between 0 and 18 months of age. Epidemiol Infect 1988; **100**: 185–91.
28. Borderon JC, Lionnet C, Rondeau C, Suc AL, Laugier J, Gold F. Current aspects of the fecal flora of the newborn without antibiotherapy during the first seven days of life: *Enterobacteriaceae*, enterococci, staphylococci. Pathol Biol Paris 1996; **44**: 416–22.
29. Rotimi VO, Duerden BI. The development of the bacterial flora in normal neonates. J Med Microbiol 1981; **14**: 51–62.
30. Goldmann DA, Leclair J, Macone A. Bacterial colonization of neonates admitted to an intensive care environment. J Pediatr 1978; **93**: 288–93.
31. Tullus T, Berglund B, Fryklund B, Kühn I, Burman LG. Epidemiology of fecal strains of the family *Enterobacteriaceae* in 22 neonatal wards and influence of antibiotic policy. J Clin Microbiol 1988; **26**: 1166–70.
32. Bennet R, Nord C-E. Relationship between anaerobic bacteria and enterobacteria in feces of newborn infants. Pediatr Infect Dis J 1987; **6**: 426.
33. Berg RD, Savage DC. Immunological responses and microorganisms indigenous to the gastrointestinal tract. Am J Clin Nutr 1972; **25**: 1364–71.
34. Mellander L, Carlsson B, Jalil F, Söderström T, Hanson LÅ. Secretory IgA antibody response against *Escherichia coli* antigens in infants in relation to exposure. J Pediatr 1985; **107**: 430–3.
35. Sarff LD, McCracken Jr GH, Schiffer MS, et al. Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns. Lancet 1975; i: 1099–104.
36. Van Camp JM, Tomaselli V, Coran AG. Bacterial translocation in the neonate. Curr Opin Pediatr 1994; **6**: 327–33.
37. Khan SR, Jalil F, Lindblad BS, Karlberg J. Early child health in Lahore, Pakistan: X. Mortality. Acta Paediatr Suppl 1993; **390**: 109–17.
38. Bhutta ZA, Naqvi SH, Muzaffar T, Farooqui BJ. Neonatal sepsis in Pakistan. Presentation and pathogens. Acta Paediatr Scand 1991; **80**: 596–601.
39. Fujita K, Kaku M, Yanagase Y, et al. Physicochemical characteristics and flora of diarrhoeal and recovery faeces in children with acute gastroenteritis in Kenya. Ann Trop Paediatr 1990; **10**: 339–45.
40. Guarino A, Guandalini S, Alessio M, et al. Characteristics and mechanism of action of a heat stable enterotoxin produced by *Klebsiella pneumoniae* from infants with secretory diarrhoea. Pediatr Res 1989; **25**: 514–18.
41. Åhren CM, Jertborn M, Herclik L, Kaijser B, Svennerholm A-M. Infection with bacterial enteropathogens in Swedish travellers to South-East Asia – a prospective study. Epidemiol Infect 1990; **105**: 325–33.
42. Jertborn M, Svennerholm A-M. Enterotoxin-producing bacteria isolated from Swedish travellers with diarrhoea. Scand J Infect Dis 1991; **23**: 473–9.
43. Neill RJ, Twiddy EM, Homes RK. Synthesis of plasmid-coded heat-labile enterotoxin in wild-type and hypertoxinogenic strains of *Escherichia coli* and in other genera of *Enterobacteriaceae*. Infect Immun 1983; **41**: 1056–61.
44. Wells CL, Maddaus MA, Jechorek RP, Simmons RL. Role of intestinal anaerobic bacteria in colonization resistance. Eur J Microbiol Infect Dis 1988; **7**: 107–13.
45. Apperloo-Renkema HZ, Van der Waaij BD, Van der Waaij D. Determination of colonization resistance of the digestive tract by biotyping of *Enterobacteriaceae*. Epidemiol Infect 1990; **105**: 355–61.
46. Apperloo-Renkema HZ, Van der Waaij D. Study of colonization resistance for *Enterobacteriaceae* in man by experimental contamination and biotyping as well as the possible role of antibodies in the clearance of these bacteria from the intestines. Epidemiol Infect 1991; **107**: 619–26.
47. Örskov F, Biering-Sörensen K. *Escherichia coli* serogroups in breastfed and bottlefed infants. Acta Path Microbiol Scand Sect B 1975; **83**: 25–30.
48. Mevissen-Verhage EAE, Marcelis JH, Guiné PAM, Verhoe J. Effect of iron on serotypes and haemagglutination patterns of *Escherichia coli* in bottle-fed infants. Eur J Clin Microbiol 1985; **4**: 570–4.