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Faecal excretion of rotavirus and other enteropathogens in newborns of the high and low socio-economic stratum in Santiago, Chile

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SUMMARY

Faecal excretion of enteropathogens was studied in newborns in their first week of life. Rotavirus was investigated in 225 neonates, of whom 107 belonged to the low socio-economic stratum (SES) and 118 to the high SES. Half of each group were delivered by caesarean section. Rotavirus was detected in 10 infants (4·4%). Eight of them had been in the same ward and excreted the same viral electrophoretype. Enteropathogenic bacteria were isolated from 8 out of 57 (14·0%) newborns. Positive cultures were equally distributed by SES and route of delivery. *Giardia lamblia* was the only parasite detected, in one infant (2·6%) of the high SES. None of the children developed symptoms. Faecal excretion of enteropathogens ended spontaneously within a week in all cases. It is suggested that the lack of symptomatology and the spontaneous termination of the faecal excretion are related to immaturity of the small intestinal mucosa, that does not allow the completion of the steps that must take place during a successful infectious event.

INTRODUCTION

Acquisition of the normal resident flora is one of the important physiological phenomena that occur in the newborn soon after birth. The sequence of events during colonization of the gut and its interaction with breastfeeding have been thoroughly reviewed (Neter & Braun, 1981; Reiter, 1984). In contrast, much less is known about the transit of enteropathogens along the intestinal lumen during the first days of life and the effects that these agents may exert. A high proportion of asymptomatic newborns have been reported to excrete rotavirus antigens in their faeces while they stayed in neonatal wards (Chrystie *et al.* 1975; Bishop *et al.* 1976; Madeley, Cosgrove & Bell, 1978; Mata, 1983; Champsaur *et al.* 1984). Whether this represents nosocomial infections by strains of rotavirus that can cause disease in suceptibles or the detection of naturally attenuated rotavirus strains is not clear. Other enteropathogens, like *Escherichia coli*, shigellae and *Giardia lamblia*, have been isolated from asymptomatic, non-hospitalized neonates born in less developed countries where levels of microbiological contamination of the environment are high (Mata, 1983). Previous studies comparing families at the extremes of the socio-economic spectrum in Santiago have shown that positive

cultures from greens and vegetables and hand imprints are significantly more frequent in the lower levels than in the higher ones (Araya *et al.* 1982).

Against this background this study was designed to detect the faecal excretion of enteropathogens in newborns during their first week of life, evaluating the influences of the route of delivery, the socio-economic stratum of their families and the environment where they spent the first days of life.

MATERIALS AND METHODS

Patients. Four maternity hospitals were selected: centres 1 and 2 were private institutions in Eastern Santiago and provided medical care to wealthy families. In both these centres, patients arrived about 12 h before delivery, were under the care of their private physicians and stayed 4–5 days in private rooms after delivery. Centres 3 and 4 were in Southern Santiago and provided medical care to communities in the low socio-economic stratum (SES). Pregnant women usually arrived at these hospitals shortly before delivery. They were attended by the hospital staff in wards with 10–12 beds, with two mothers sometimes sharing one bed. Mothers who had normal, uneventful vaginal deliveries and whose newborns were in satisfactory condition stayed in the hospital for 12–48 h, after which they were transferred to puerperium wards at another centre about 15 kilometres away. In these puerperium wards they completed 3 days before being discharged.

A total of 280 mothers (70 from each centre) were contacted within 24 h of delivery, the purposes of the study were explained to them and they were invited to participate. Those who agreed to do so then answered a questionnaire designed to assess their SES on Graffar's scale (Graffar, 1957; Alvarez, Wurgaft & Salazar, 1982). They were also questioned about the quality of their houses (mainly on the availability of intradomiciliary water and disposal of excreta), their clinical history prior to and during this pregnancy and about any illnesses diagnosed in the mother and the newborn during the first post-partum week. Information collected about the newborns included birth weight and length, gestational age, Apgar scores 1 and 5 min after birth and breastfeeding. Half the children had been born by spontaneous vaginal route and the other half by caesarean section.

Rotavirus. Five faecal samples, each of about 2 g, were obtained between days 1 and 7 of life. The specimens were put into vials, cooled on ice and taken to the virology laboratory. On arrival they were diluted fivefold in 50 mM Tris-HCl buffer, homogenized and stored at −20 °C. The samples were analyzed within 4 days using three techniques: (1) an enzyme immunoassay (EIA) (Rotazyme®, Abbott Diagnostic Laboratory, Chicago, IL. USA) carried out following the manufacturer's instructions. (2) electron microscopy (EM) in which a 1·5 ml aliquot was centrifuged at 3000 rpm for 30 min and the viral particles precipitated from the supernatant by adding an equal volume of a saturated solution of ammoniun sulphate in distilled water. This was allowed to stand for 1 h and the precipitate sedimented at 13000 rev min^{-1} for 30 min. The supernatant was drained and the sediment redissolved in 50 μl of distilled water. Approximately 15 μl were placed on a Formvar and carbon coated 200 mesh copper grid. The grid was dried at room temperature, rinsed three times in distilled water, stained with 3% ammonium molybdate for 45 s and dried again. This procedure is also suitable for calici-,

astro-, corona- and adenoviruses. (3) RNA polyacrylamide gel electrophoresis (RNA-PAGE). Viral RNA was extracted from the faecal suspension using the method described elsewhere (Laemmli, 1970; Spencer, Avendaño & Carcia, 1983). The initial detection was carried out in small polyacrylamide gels without a stacking gel and the sample was mixed with a solution containing agarose. This allowed the samples to be electrophoresed in a horizontal electrophoresis chamber at 100 V for 60 min. The gel was then stained using a silver nitrate solution and developed until a satisfactory degree of contrast was obtained. The reaction was stopped by the addition of 10% acetic acid.

For the purpose of this study a sample was considered positive only when all three methods detected rotavirus.

Enteric bacteria. The presence of enteropathgenic bacteria was studied in a subgroup of 60 newborns evenly distributed between the four centres. Half of these infants had been born by the vaginal route and the remainder by caesarean section. Five faecal samples were obtained during the first 7 days of life. Each set of samples included two rectal swabs, moistened in Stuart's transport medium and cultured for enteropathogenic *Escherichia coli* (classic serotypes, toxigenic (LT and/or ST) and invasive strains) shigella, salmonella & *Campylobacter jejuni/coli* (Sereny, 1955; Gianella, 1976; Butzler & Skirrow, 1979; Martin & Washington, 1980; Ristaino, Levine & Young, 1983).

Enteroparasites. Three faecal samples were collected, one every second day, during the first week of life. These specimens were processed by Burrow's technique (Burrows, 1967).

Repeated daily faecal samples were obtained from all children who had positive results until two consecutive samples were negative regardless of the age of the infant.

RESULTS

Of the 225 newborns studied, 118 belonged to high SES (Graffar levels 1, 2) and 107 to low SES (Graffar levels 5 and 6); 61 of the 118 and 56 of the 107 were born by the vaginal route. The rest were born by caesarean section. Mean ($\pm$S.D.) gestational age, birth weight and birth length were $39{\cdot}1 \pm 1{\cdot}1$ weeks, $3399 \pm 403{\cdot}4$ g and $49{\cdot}95 \pm 1{\cdot}85$ cm for those from the high SES and $39{\cdot}4 \pm 1{\cdot}3$ weeks, $3333 \pm 407{\cdot}3$ g and $49{\cdot}94 \pm 2{\cdot}2$ cm for the newborns of the low SES. All pregnancies were uneventful. The Apgar score at 5 min ranged between 8 and 10 in all infants. In 39 (17%) newborns, 21 from the high SES and 18 from the low SES, a clinical abnormality was diagnosed during the follow-up period; in 87% this was perinatal jaundice. No differences were observed between Centres or route of delivery. Since children from the low SES were discharged between days 3 and 4 of life while nearly all their peers from the high SES remained in the hospital for 5 days, the results were also analysed according to the place where the samples had been collected, either at hospital or at home. No differences between these two groups were observed. All children remained asymptomatic, breastfed normally and weight increments were adequate during the observation period.

At least one enteropathogen was detected in 21% of the newborns (Table 1). The frequency of detection of rotavirus differed according to the technique used (Table 2); while 26 were positive by EIA only 10 cases had positive results by all

Table 1. *Percentage of detection of enteropathogens in newborns by socio-economic stratum*

Enteropathogen	No. of specimens studied	Socio-economic stratum (%) High	Low	Total (%)
Rotavirus	225	1 (0·4)	9 (4·0)	10 (4·4)
Bacteria	57	4 (7·0)	4 (7·0)	8 (14·0)
Parasites	39	1 (2·6)	0 (0)	1 (2·6)

Table 2. *Rotavirus detected by EIA*, RNA–PAGE† and EM‡ in 225 newborns during their first week of life*

Method	High SES Vaginal delivery	High SES Caesarean section	Low SES Vaginal delivery	Low SES Caesarean section	Total number of cases with positive results
EIA only (+)	8	7	4	7	26 (66·7%)
RNA–PAGE and EM	0	0	1	2	3 (7·7%)
EIA, RNA–PAGE and EM	1	0	2	7	10 (25·6%)

† RNA–PAGE, RNA detected by polyacrylamide gel electrophoresis.
‡ EM, electron microscopy.
* EIA; enzyme immunoassay.

three techniques. Table 3 shows that most of the positive EIA reactions were weak and that neither the route of delivery nor the SES influenced the frequency of detection. Also that positive results by EIA were obtained from the first post-partum day. In contrast, when rotavirus was detected by all three methods, positive results were obtained only after the third day of life (Table 4). Faecal excretion of rotavirus ceased spontaneously in all neonates in 3–4 days. Nine of the newborns whose faeces were positive by all three methods had been born in centre 4 between June and August 1985. All had developed jaundice shortly after birth and had been transferred to a special ward for phototherapy. Of the total of 34 newborns who developed jaundice during this study only the ten mentioned above excreted rotavirus. As nosocomial infection was suspected, faecal specimens were collected from all the neonates undergoing treatment in this phototherapy ward. Of the 15 additional newborns studied, 13 were positive by all three methods. 'Short' electrophoretypes were detected in all cases (Fig. 1). Electrophoretypes 1–20 were found in the newborns included in this study. All the electrophoretypes, except that of case 1, belonged to children who had received treatment at the phototherapy ward in centre 4. Electrophoretypes 21, 22 and 23 were obtained from infants who did not participate in this study but who were in the same phototherapy ward. All the newborns excreted a single, homogenous strain of rotavirus, although some additional unusual bands were also visualized in a few cases (16 and 19 in Fig. 1). Fig. 2 shows another set of 'short' and 'long' electrophoretypes, of which only no. 33 came from a neonate included in this study. The remaining were obtained from infants older than 3 months of age who

Table 3. *Day of detection, route of delivery and socio-economic level of newborns who were positive only by EIA*

Patient no.	Result by EIA	Day of detection	Route of delivery*	Socio-economic level
001	+	6	V	High
012	+	4	C	Low
014	+	4	V	Low
033	+	5	C	High
035	+	3	C	Low
040	+	5	V	High
043	+	7	C	Low
049	+ +	3	V	High
052	+	2	C	High
060	+	4	V	Low
064	+	4	V	Low
065	+	7	C	Low
066	+	3	C	High
068	+	4	C	Low
074	+	1	V	High
076	+	1	V	High
077	+ + + +	1	C	High
082	+ + +	6	C	High
	+ +	7	C	High
087	+	4	V	High
089	+	6	V	High
091	+	3	V	High
097	+ +	2	V	Low
105	+	6	C	High
140	+ +	1	C	High
194	+	7	C	Low
206	+	5	C	Low
	+	6	C	Low

* V, vaginal delivery, C, caesarean section.

were admitted at the same time to a pediatric hospital in Santiago for acute diarrhoea (cases 25 and 34–43). Their asymptomatic, age-matched controls are shown as cases 24–32. Fig. 3. illustrates the results obtained by electron microscopy. All types of particles were detected, including complete, double-shelled infective particles. Small round viral particles were observed in one child.

Because the immune status is an important factor influencing the host's response, cord blood samples were analysed for antirotavirus antibodies in a subgroup of 53 neonates (28 from the high and 25 from the low SES). An enzyme immunoassay was developed to detect rotavirus antigens using a mixture of long and short electrophoretypes. Several experiments used to determine the optimal concentration of viral antigens and conjugated antibody at which the enzymatic reaction correlated with the concentration of rotavirus antibodies in the sample. After serial dilution of hyperimmune serum, three dilutions for each serum sample were selected: 1/2500, 1/5000 and 1/10000 with the results shown in Fig. 4. They are expressed as a percentage of the reaction obtained with a known standard, hyperimmune serum (obtained from NIH, Bethesda, MD, USA), which was given a value of 100%. None of the samples showed reactions below 15% of the standard.

Table 4. *Newborns who had rotavirus detected by EIA, RNA–PAGE and EM*

Patient no.	EIA*	RNA–PAGE	EM	Day of positive excretion	Route of delivery†	Socio-economic stratum
009	+ +	+	+	4	V	High
013	+ + + +	+	+	5	C	Low
	+ + +	+	+	6		
150	+ + + +	+	+	7	C	Low
153	+	+	+	5	V	Low
	+ +	+	+	6		
	+ +	+	+	7		
194	+	+	+	6	C	Low
	+	−	−	7		
196	+ + + +	+	+	5	V	Low
	+	+	+	6		
	−	+	+	7		
203	−	+	+	4	C	Low
	+ + + +	+	+	5		
	+ + + +	+	+	6		
	+ + +	+	+	7		
204	+ + +	+	+	6	C	Low
	+ +	+	+	7		
206	+	−	−	5	C	Low
	+	−	−	6		
	+ + +	+	+	7		
208	+ +	+	+	4		
	−	+	+	5		

* + − + + + +, Positive. No. of plusses indicates level of positivity. −, Negative result.
† C, caesarean section; V, vaginal delivery.

Samples from the high SES were mostly between 15 and 35% while none of the specimens from the low SES yielded results below 35%. The antibody levels were high in all cases if the dilutions used for the analysis are taken with account but the identification of rotavirus in the faeces was not related to the level of antibodies detected in the cord blood.

Enteropathogenic bacteria were isolated in 8 (14%) of the 57 newborns studied: 5 *E. coli* of classic serotypes, 1 LT-producing *E. coli*, 1 *Salmonella paratyphi* and 2 *Campylobacter coli*. One neonate of the low SES who had been born by caesarean section excreted *E. coli* strain O 18.ac.K77 and *Campylobacter coli* simultaneously. Of the 8 children with positive results, 4 came from the low SES and the same number had been delivered by caesarean section. All isolates were obtained after the third day of life and ceased spontaneously within 5 days.

Only 39 mothers obtained the three faecal samples required for parasitological studies. Of these, only one infant, from the high SES and delivered vaginally, excreted *Giardia lamblia*. A second faecal specimen and one from his mother were both negative from parasites.

DISCUSSION

This study shows that enteropathogens are found in the gastrointestinal tract from very early in life, and starts when the newborns are still in hospital. Previous studies on the faecal excretion of enteropathogens in asymptomatic infants

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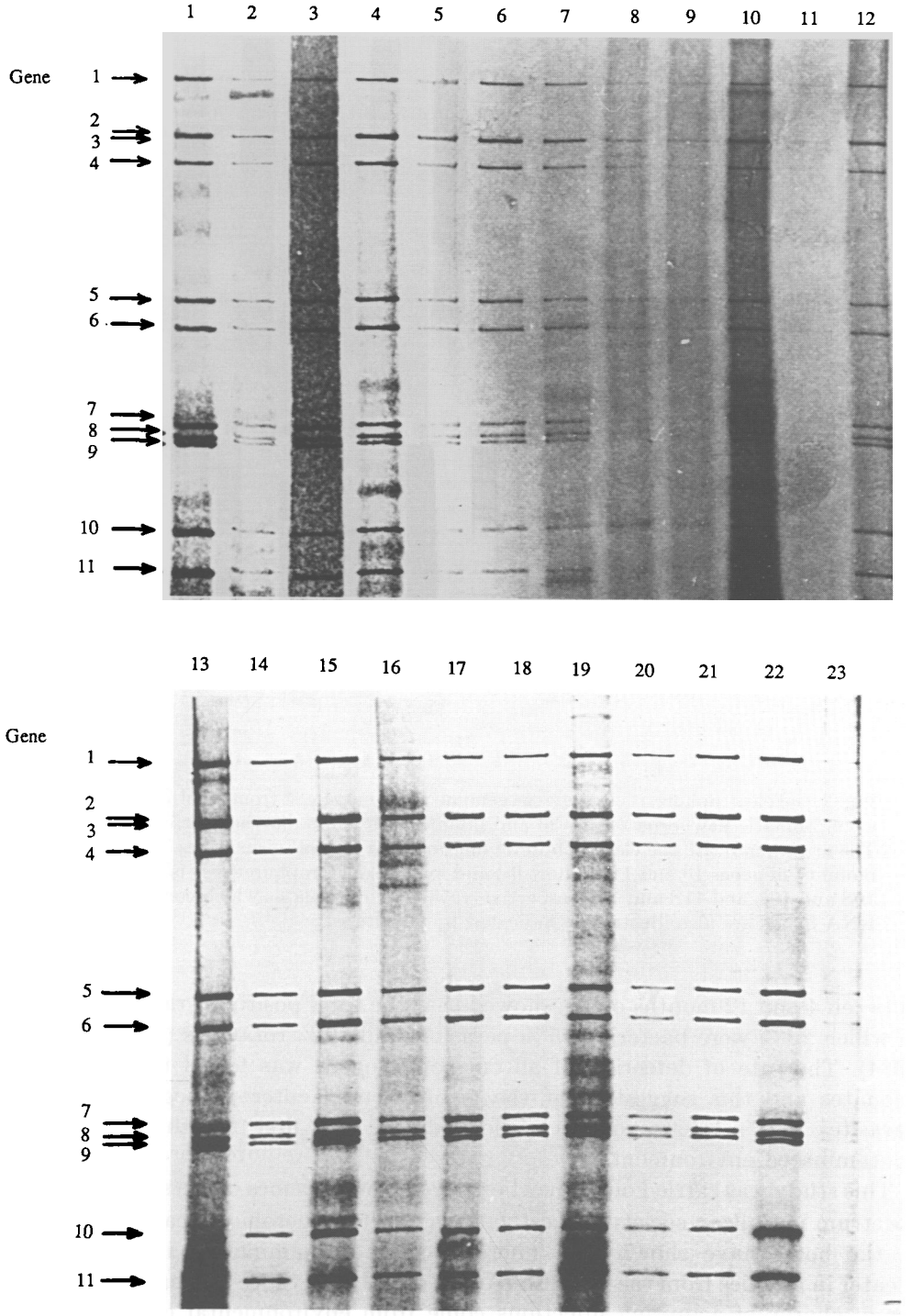

Fig. 1. Gel electrophoresis of rotavirus genome RNA extracted from stool samples from newborns of low and high socio-economic stratum. (1–20). Samples 21–23 came from newborns in the phototherapy ward. The arrows indicate the electrophoretic migration of the viral genes.

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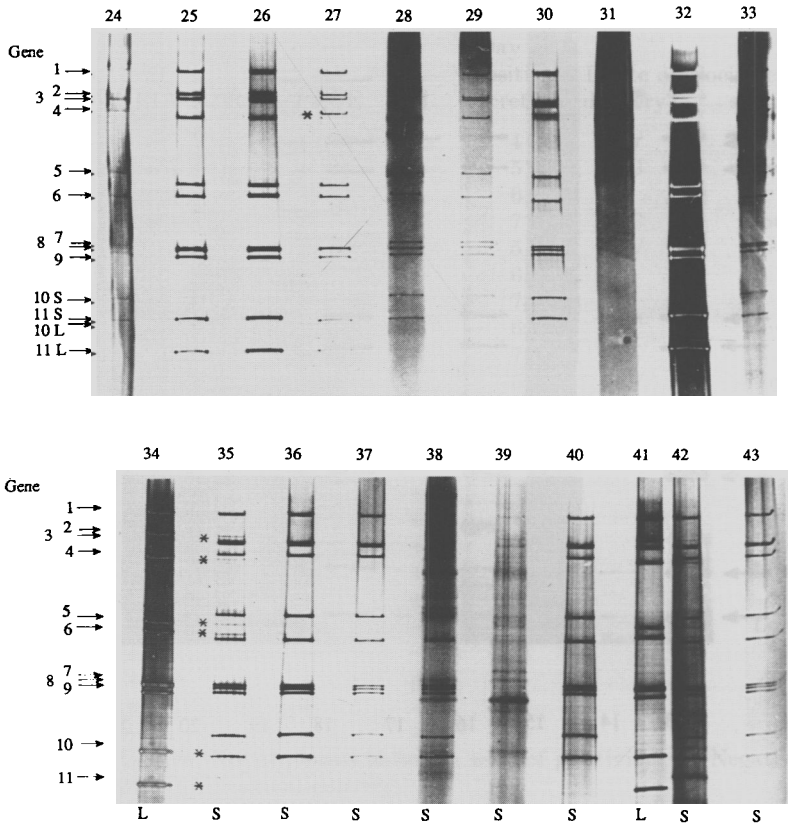

Fig. 2. Gel electrophoresis of rotavirus genome RNA extracted from stool samples from asymptomatic newborns 24, 26–33 and infants with acute diarrhoea (25 and 34–43). The arrows indicate the electrophoretic migration of the rotavirus genes. The distinct mobility of genes 10 and 11 in short (S) and long (L) electrophoretypes is indicated as 10S and 10L and 11S and 11L respectively. Some viral isolates which contain 'extra' RNA bands are also illustrated, indicated by an asterisk.

between 4 and 12 months of age showed that the total positivity rate was 36·5%, of which 20% were bacteria, 6·5% parasites and 10% rotavirus (Figueroa *et al.* 1984). The rate of detection of all enteropathogens was found to be lower in neonates and this suggests that the acquisition of enteropathogenic bacteria, parasites and rotavirus occurs rapidly during the first months of life in a contaminated environment.

This study was carried out in newborns from the extremes of the socio-economic spectrum in Chilean society. Investigations into the microbiological environment in the home have shown that microbiological contamination is significantly greater in families from the low SES (Araya *et al.* 1982). Since the main risk factors for newborns of acquiring infections come from environmental contamination, neonates from a low SES should have been at greater risk of contact with potential enteropathogens after they have been discharged from hospital. However, analysis of the results has shown that contact with enteropathogens began when

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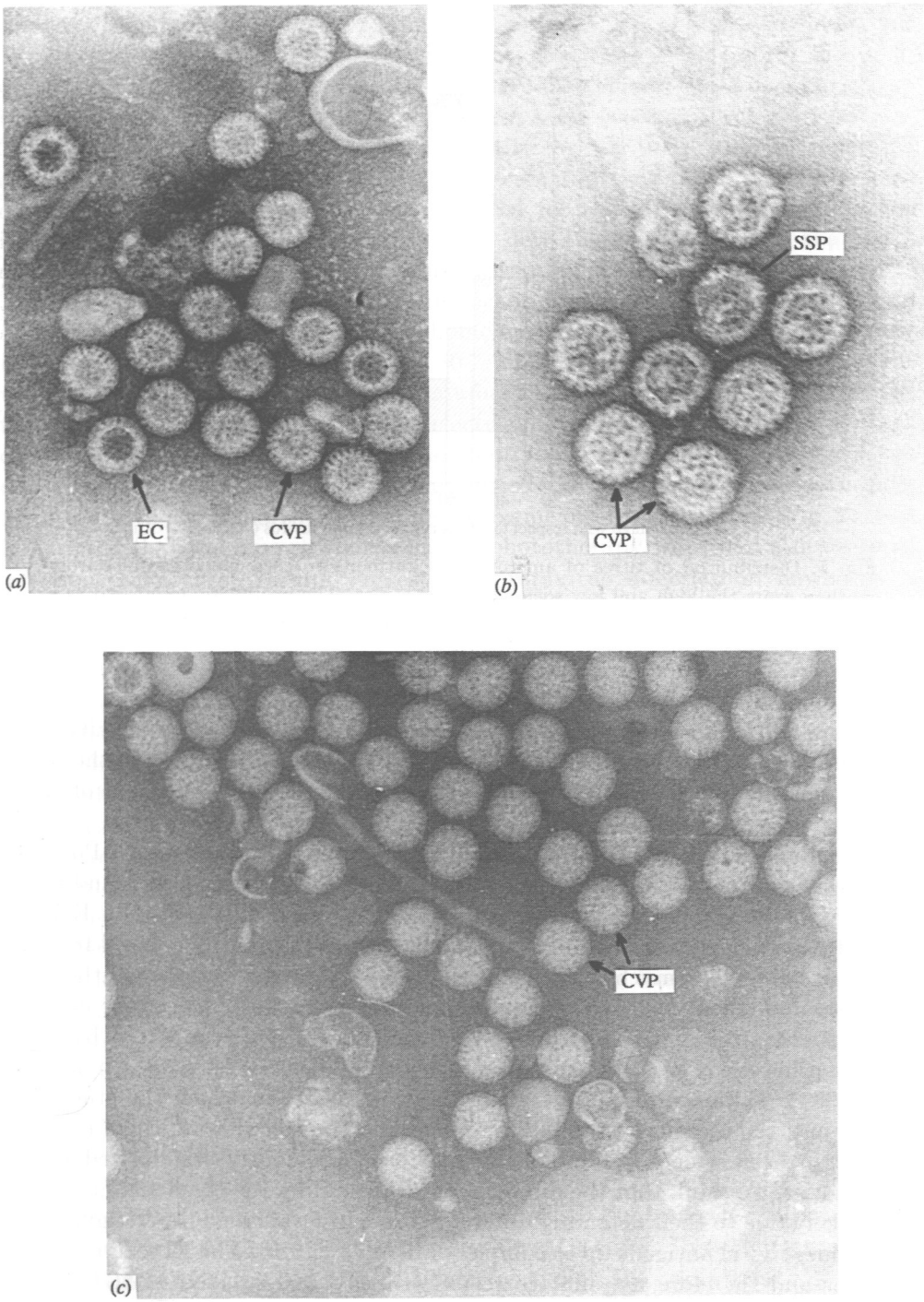

Fig. 3. Electron micrographs of rotavrius particles detected in case 013 on the 5th (A) and 6th days of life (B) and in case 150 on day 7 of life (C). All samples are stained with 3% ammonium molybdate showing complete virus particles (CVP), single shelled particles (SSP) and empty capside (EC).

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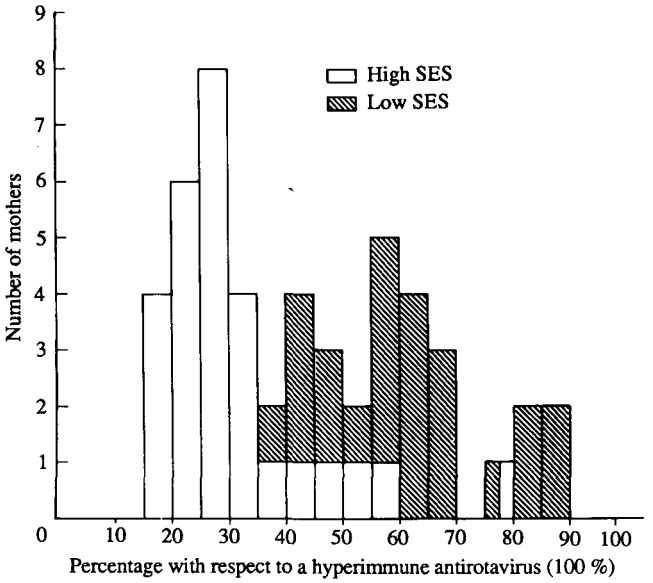

Fig. 4. Distribution of titres of antirotavirus antibody in cord blood samples from mothers from the high and low socio-economic strata. The results are expressed as a percentage of the reaction observed with a hyperimmune antirotavirus serum which was an arbitary value of 100.

they were still in hospital, but there were no differences between positivity rates obtained from samples collected at home or at hospitals, regardless of the socio-economic level. Nor did the route of delivery correlate with the incidence of faecal excretion of enteropathogens.

The rate of detection of rotavirus depended on the technique used. 'Positive' cases were arbitrarily defined as those in whose stools rotavirus was found by all three methods used. If the children whose stools were positive only by EM and RNA–PAGE are included in the analysis, the positivity rate is increased from 4·4 to 5·7%, but is still much lower than the rates published by other authors. In contrast, if all those positive by any metres are included, 17·3% of the children excreted rotavirus and this figure is comparable with those reported by other authors (Chrystie *et al.* 1978; Bishop *et al.* 1976; Madeley *et al.* 1978; Champsaur *et al.* 1984). Taking into consideration the time of life in which the study was carried out, the number of samples obtained and the fact that all three methods used have been widely validated and had been previously standarized in our laboratories, we think that the different results found by the three techniques are most likely due to their detecting different stages of viral assembly, ranging from unorganized viral antigens up to complete infective virions. The EIA detects viral antigens and therefore may not correlate with EM and/or RNA–PAGE, which detect different properties. Positive results were obtained during the first 3 days of life only by EIA and nearly all were weak reactions. These may be due either to nonspecific reactions or to detection of viral products from abortive infections. Experiments to evaluate the specificity of this reaction (boiling and titration of the samples and plotting concentration/reactivity curves) did not suggest that the

reaction was nonspecific. Tests on the same samples using EIA kits from other sources yielded entirely similar results. Furthermore, strongly positive reactions to two cases (077 and 082 in Table 3) suggests that the results are indeed due to the detection of nonorganized viral antigens. If this is correct, it is logical to expect that EM and RNA–PAGE remain negative in these cases.

The majority of infants who were positive by all three methods excreted complete viral particles. That these results were always obtained after the third day of life may be due to the time required for the virus to multiply in the intestinal epithelium and is consistent with detection by EIA during the first 3 days being due to incomplete infectious cycles.

Nine out of the 10 positive cases were born in centre 4 within a 3 month period and were all on one ward. A single, homogeneous, short electrophoretype was isolated from these children and from 80% of all the newborns who were on the ward at the same time (Fig. 1). Other studies by one of the authors (E.S.) at the time of this study showed that several electrophoretypes were present in Santiago children with diarrhoea. That the strain identified in the neonates at centre 4 was not a naturally attenuated one is supported by the fact that other infants admitted with acute diarrhoea excreted the same electrophoretypes (Fig. 2). If it is accepted that the nine newborns on the phototherapy ward had a nosocomial infection, then these results suggest that rotavirus infection in newborns is infrequent during the first week of life unless special circumstances occur.

One factor that may moderate the effects of rotavirus infection is maternal immunity. Measurements of antirotavirus antibodies in cord blood sera from a subgroup of neonates from both SES showed high titres in all of them and suggests that rotavirus is widespread in Santiago as a whole.

It is difficult to explain why all the newborns who excreted enteropathogens remained asymptomatic and why this excretion ceased spontaneously within a few days. This is specially remarkable in the case of *Giardia lamblia*, an enteroparasite known to colonize the intestine of the host and remain there for long periods (Solomon, 1982). The outcome of infection by enteropathogens in newborns may be related both to the properties of the agent and to the characteristics of the neonatal intestinal mucosa. The neonatal small intestinal mucosa has been described as immature in its ability to digest some nutrients (Lebenthal & Lee, 1983; Mathews 1983) and in its immunological responses (Silverstein, 1977). This 'immaturity' may also affect the ability of the small intestinal mucosa to allow successful replication to take place.

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