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Epidemiology of Pharyngeal Colonization of Infants with Aerobic Gram-Negative Rod Bacteria

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By using a selective medium, pharyngeal colonization with gram-negative rod (GNR) bacteria was determined in a cohort of 49 normal infants monitored from birth to 6 months of age. Culture swabs were diluted in 1 ml of saline for quantitation. The prevalence of GNR in the first 72 h of life was 8% and rose to 29% during the first month, 52% at 2.5 months, 67% at 4.5 months, and 62% at 6 to 7 months. Colonization was with substantial numbers of organisms, generally >100 colonies per ml and frequently >1,000 colonies per ml. The most common species were *Klebsiella* species, *Escherichia coli*, *Enterobacter* species, and *Acinetobacter anitratus*. Fewer infants who were breast fed rather than formula fed at the time of culture harbored GNR (26 versus 45%, $P < 0.05$). The point prevalence of pharyngeal GNR colonization in our special care nursery was 12 of 47 (26%), which was found to be similar to that of age-matched normal infants. GNR carriage in normal infants does not appear to be a residual of organisms acquired at birth, and interpretations of GNR carriage in ill or hospitalized infants should be evaluated by comparison with these data in healthy infants.

Studies of pharyngeal bacterial flora have been performed on defined populations both to obtain information about normal human microflora and to evaluate populations with a high susceptibility to respiratory infections. While gram-negative rod (GNR) species are not usually considered normal pharyngeal flora, a number of studies have demonstrated GNR colonization in normal subjects. The majority of studies of normal subjects have been of adults (5, 7, 8, 12), but there are some pediatric data (1, 4). With increased interest in susceptibility to nosocomial infections, there have been studies of GNR carriage in hospitalized adults (5, 12) and infants (4) and comparisons between hospitalized and normal adults (5, 12). As yet, there have been no published longitudinal studies of GNR carriage in normal infants which compare carriage rates with those of age-matched hospitalized infants.

This report is of a prospective longitudinal study of GNR colonization of normal infants from birth through 6 months of age. The selective culturing method is similar to methods used in previous studies by other investigators, but this is the first such study to employ a quantitative method of plating specimens. We also cultured samples from infants of similar ages in a newborn intensive care unit by using the same culture methods.

MATERIALS AND METHODS

Patients. The infants in this study were all born at Yale-New Haven Hospital, New Haven, Conn., and were cared for in the normal newborn nursery. They were excluded if they had spent more than the 6- to 8-h holding period in the newborn special care unit, if they were ill or premature (requiring admission to the special care unit for prematurity), if they had been treated with antibiotics, or if they had major congenital anomalies or any anomalies of the head and neck. The parents of an eligible infant were approached for the study if they indicated that regular pediatric care would be either at the Primary Care Center at Yale-New Haven Hospital or at Pediatric and Adolescent Medicine Professional Corporation (PC) of Orange, Conn., the two care centers involved in the study. Parents of eligible newborn infants were given a consent form approved by the Human Investigation Committee of Yale-New Haven Hospital and the elements of the study were discussed with them. Samples from enrolled infants were cultured by one of us (R.L.D. or R.S.B.) within 72 h of birth and at each clinic visit by a staff member of the clinic. Two standard questionnaire forms were also used, one at the initial contact in the newborn period and another at each subsequent culture visit. The questions involved general health and interval health history, feeding history, use of antibiotics, and illness in the home. The culture protocol called for an initial culture within 72 h of birth and others at regular health-care visits at approximately 2, 6, 12, 18, and 26 weeks of age, as well as at any visits for illnesses. For data analysis, culture samples taken during the following time periods were pooled and designated as periods A to E, where A was 0 to 72 h of age, B was 1 to 3 weeks of age, C was >3 weeks to 2.5 months of age, D was >2.5 to 4.5 months of age, and E was >4.5 to <7 months of age.

Infants were considered to be breast fed if they were receiving one or more breast-milk feedings per day at the time of culture.

Culture methods. A Culturette-Brand minitip swab was placed on the upper posterior oropharynx and held there for 1 to 2 s until saturated. It was replaced in the sleeve until ready to be plated. The culture obtained in the newborn period was plated within 1 to 2 h of being obtained. Cultures at later clinic visits were generally plated 6 to 18 h after being obtained. The Culturette allows the swab to remain in modified Stuart medium, in which the bacteria are preserved but do not multiply.

Two swabs were cultured from each subject. One was inoculated directly onto a MacConkey agar plate by rolling the swab over the whole plate. For quantitation, the other swab was extracted thoroughly into 1 ml of sterile saline. By using a quantitative loop, 0.01 ml was plated onto another MacConkey agar plate. Another 0.01 ml was plated onto a Mueller-Hinton agar plate. This procedure provided us with

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both a qualitative determination of GNR prevalence (maximum sensitivity determined by the presence or absence of GNR on the direct-swab plate) and a quantitative measure of GNR density over a broad range of concentrations (2).

If any GNR species was recovered on a MacConkey agar plate it was recorded as positive. For quantitation, the number of colonies was recorded if colonies only appeared on the direct-swab plate. The concentration, in CFU per milliliter, was recorded when there were colonies on the plate inoculated with the quantitative loop. Mueller-Hinton agar is a general medium and was used to demonstrate the presence of bacterial flora in samples in which no GNR were recovered. While a few samples from newborn infants showed no growth on Mueller-Hinton agar plates, all samples from infants >72 h had growth, demonstrating that a sample was obtained. In some cases, samples were taken at times when it was anticipated that more than 18 h would elapse before the sample was plated. These cultures were directly plated on MacConkey agar and were not included in the analysis of the quantity of bacteria in pharyngeal cultures.

Some subjects had more than one culture taken during one of the time periods. For the purpose of enumeration of GNR species, if more than one culture had GNR recovered, only one culture (arbitrarily, the second) was counted. If one was positive during the time period and the other(s) was negative, the positive culture was recorded. If none were positive for GNR, the culture was counted as negative once for the time period.

All agar plates were incubated at 37°C and held for up to 48 h to determine whether there was growth on MacConkey agar plates. If there was growth, representative colonies were isolated and identified by species in the Clinical Microbiology Laboratory at Yale-New Haven Hospital by using standard methods for identification (6). It should be noted that these methods were designed to achieve the objective of recovering major potential GNR pathogens, including members of the family *Enterobacteriaceae*, *Pseudomonas* species, and *Acinetobacter anitratus*. Isolation of fastidious GNR or anaerobes was not attempted in this study.

RESULTS

Forty-nine infants were enrolled in the study, and samples from them were cultured within the first 72 h of life. Twelve infants received their regular care at the Pediatric and Adolescent Medical PC of Orange, and the rest received their care at the Primary Care Center of Yale-New Haven Hospital. Due to missed appointments and two dropouts from the study, samples from 21, 21, 36, and 29 subjects were cultured during time periods B, C, D, and E, respectively. Samples from 11 subjects were cultured in all five time periods, samples from 8 subjects were cultured in four time periods, samples from 12 subjects were cultured in three time periods, samples from 12 subjects were cultured in two time periods, and six subjects had samples taken only for the initial culture. There were 156 culture samples taken during the whole study, counting only one culture per time period per patient. All infants in the study remained healthy throughout the study period, and none of the infants was hospitalized.

Twenty-seven percent of all samples were taken from infants who were breast fed at that time. Of the infants cared for at the Pediatrics and Adolescent Medicine PC of Orange, 58% (7 of 12) were breast fed for some period, while 19% (7 of 37) of the infants cared for at the Primary Care Center of Yale-New Haven Hospital were breast fed.

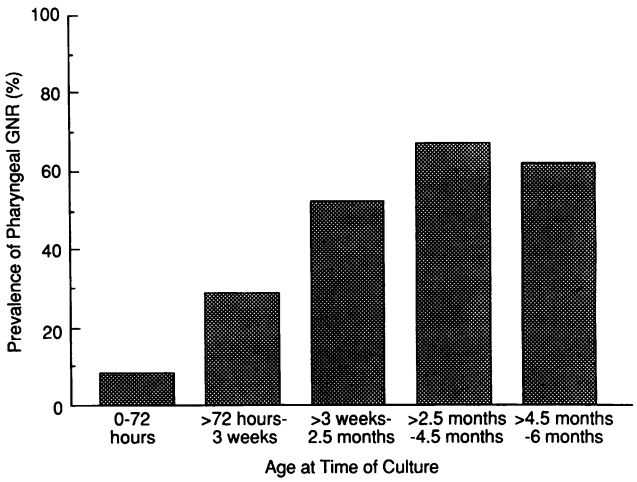

FIG. 1. The prevalence of pharyngeal GNR colonization of infants from birth through 6 months of age. The ages of infants in the five time periods (from left to right, A to E) are indicated along the *x* axis.

Prevalence of pharyngeal colonization with GNR. The prevalence of pharyngeal colonization with GNR from birth through 6 months of age is shown in Fig. 1. It can be seen that the rate of carriage rises from 8% in the first 72 h to 29% in period B, and then to 52, 67, and 62% in periods C, D, and E, respectively. Of the 49 patients who had at least one sample cultured, 34 (69%) had at least one culture that grew GNR during the study, and 15 (31%) never had a positive culture for GNR. The prevalence of GNR isolation was significantly lower in periods A and B (but not C) than in the succeeding time periods ($P < 0.05$ by chi-square analysis).

Species of GNR encountered are shown in Table 1. Of the five most commonly encountered species, four are glucose-fermenting members of the *Enterobacteriaceae*. *A. anitratus* is a nonfermenter. These five represent 69% of the pharyngeal isolates in the study. The number of subjects carrying each species at any time during the study was similar to the number of isolates shown in Table 1. Twenty-one subjects carried *Klebsiella pneumoniae* at some time during the study, 16 had *Enterobacter cloacae*, 12 had *E. coli*, 7 had *A. anitratus*, 5 had *Klebsiella oxytoca*, 2 each had *Pseudomonas aeruginosa* and *Pseudomonas putida*, and the remaining species were the same as the total number of isolates shown in Table 1.

Quantitation of GNR in pharyngeal cultures. As noted in Materials and Methods, some specimens could only be cultured by the care givers on site using only a direct swab, because they would have to be held for more than 18 h before reaching the study laboratory. These specimens were eliminated from the analysis of organism quantitation unless they had fewer than 10 colonies on the plate. Most of the specimens were plated according to the protocol, and the results for the five most common species, which were responsible for 69% of isolates, are shown in Table 2. The data shown in this table exclude the 24 isolates (32%) that were not plated quantitatively. Of the 12 plates with <10 colonies of a particular species per direct swab, eight also had other species in a concentration of >100 colonies per ml.

Association of breast feeding and GNR colonization. In all time periods except period B, the prevalence of GNR colonization was lower among infants currently breast feeding. In time period A, breast-fed infants had a 7% prevalence

TABLE 1. Species of GNR recovered from pharyngeal cultures of normal infants from birth to 6 months of age

Species	No. of isolates[a] during time period[b]:					
	A	B	C	D	E	Total (%)[c]
Klebsiella pneumoniae	1	4	6	9	7	27 (25)
Enterobacter cloacae	1	1	5	7	6	20 (18)
Escherichia coli	2	1	5	7	6	16 (15)
Acinetobacter anitratus		1		7		8 (7)
Klebsiella oxytoca			1	2	2	5 (5)
Acinetobacter lwoffii			1	2	1	4
Enterobacter agglomerans		1		2	1	4
Pseudomonas aeruginosa			1	2	1	4
P. putida	1	1	1			3
P. maltophilia			2		1	3
Serratia marcescens			1	2		3
Enterobacter aerogenes			2			2
Hafnia alvei				1	1	2
Citrobacter freundii				2		2
Enterobacter gergoviae				1	1	2
Pseudomonas fluorescens			1			1
Proteus mirabilis	1					1
Enterobacter sakazakii					1	1
Kluyvera species					1	1
King-Weaver group 10				1		1

[a] Each species was counted only once per subject for each period. There were 49 subjects and a total of 156 cultures.

[b] A, 0 to 72 h; B, 1 to 3 weeks; C, >3 weeks to 2.5 months; D, >2.5 to 4.5 months; E, >4.5 to <7 months.

[c] (No. of isolates of that species)/(total no. of isolates) × 100.

of GNR, and non-breast-fed infants had a 9% prevalence. In time period B, GNR prevalence was 29% for both groups; in time period C, it was 40% (breast fed) and 64% (non-breast fed), in time period D it was 50% (breast fed) and 71% (non-breast fed), and in time period E it was 0% (breast fed) and 65% (non-breast fed). The rate of GNR recovery in all cultures at all time periods was 26% (11 of 42) for currently breast-fed infants versus 45% (51 of 114) for non-breast-fed infants. (In this analysis, each subject's culture for each time period was counted.) This difference was significant (χ^2 = 4.40; $P < 0.05$). Of infants who had some breast feeding, 64% had at least one culture with GNR. This was similar to 71% of infants who never had breast feeding who had at least one culture with GNR.

Prevalence of GNR in hospitalized infants in the newborn special care unit. In order to compare the pharyngeal colonization of normal infants with hospitalized infants, on a single day at approximately the midpoint of the prospective longitudinal study, we performed a point-prevalence study of pharyngeal colonization with GNR in the newborn special care unit. Each of the infants in the newborn special care unit at Yale-New Haven Hospital had a pharyngeal culture performed and plated by the same methods used for the normal infants. Of the 47 infants cultured, 20 infants were in an acute illness ward with intensive support and monitoring (room 1) and 27 were in a separate section in which infants were less acutely ill and were primarily stable prematures being held in the hospital to gain more weight (room 2). The prevalences of pharyngeal GNR were 15% in room 1 and 33% in room 2. The overall prevalence was 26%. Of the 12 infants who harbored GNR, 7 harbored two species of GNR. The species isolated were *E. coli* (8 isolates), *K. pneumoniae* (4 isolates), *P. aeruginosa* (3 isolates), *K. oxytoca* (2 isolates), and *Serratia marcescens* (2 isolates). Of the seven infants who had two species of GNR, six had *E. coli* as one of the species.

It was noted that infants in room 2 had a higher prevalence of GNR than those in room 1 and were older. Overall, the colonized infants were older than the noncolonized infants. The mean age of colonized infants was 45 ± 18 days, while that of noncolonized infants was 16 ± 20 days. This difference was significant ($P < 0.001$ by the two-tailed t test for two means). One outlier, a noncolonized infant 330 days of age, was excluded from these calculations. The length of hospitalization was similar to age and was also significantly greater for colonized infants. The median age of colonized infants was 41 days, and for noncolonized infants it was 7 days. This difference was also significant ($P < 0.01$ by the median test [10]).

TABLE 2. Quantitation of the five most common GNR isolates in pharyngeal cultures from normal infants

Species	No. of cultures with the following no. of colonies per ml:				
	<10 (on direct swab only)	10^2–10^3	10^3–10^4	10^4–10^5	≥10^5
Klebsiella pneumoniae	4	10	3	2	1
Enterobacter cloacae	5	7	2	0	0
Escherichia coli	0	1	1	3	3
Acinetobacter anitratus	3	1	2	1	0
Klebsiella oxytoca	0	2	0	0	1

DISCUSSION

Previous studies of pediatric patients have not compared the prevalence of pharyngeal GNR colonization of hospitalized patients with that of healthy children, nor have age-specific GNR colonization rates for the first 6 months of life been reported. Barrie et al. studied pharyngeal colonization with *E. coli* in children less than 2 years of age (1). They found a peak prevalence of 40% at 21 days of age, with a fall after that age to 0 to 13% in year 2 of life (1). Rotimi and Duerden in Sheffield, England studied the bacterial flora during the first week of life (9). While they did not take culture samples from the throat, the prevalence of colonization with members of the *Enterobacteriaceae* in the mouth between days 2 and 3 of life was 4 to 13% (9), which was similar to our finding in period A (8%). Goldmann and associates have studied colonization rates in neonates in an intensive care environment (4). Looking at *Klebsiella*, *Enterobacter*, and *Citrobacter* species, they found a pharyngeal colonization rate of 22%. It was higher in those who had received more than 3 days of antibiotic treatment (4). The prevalence rate of 26% in hospitalized infants in our study is similar to the rate of 22% reported by Goldmann et al. and is also similar to the rate we saw in normal infants of the same age. Thus, the data in our study are similar to those published for normal and hospitalized infants but put them in a broader context because of the sequential culturing and the comparison of normal and hospitalized infants by using the same technique. The age of the infant appears to be the primary factor associated with GNR colonization.

Rates of pharyngeal GNR colonization of infants can be compared with rates in other populations. In 100 randomly selected healthy subjects, mostly adults, the colonization

rate was 18% with members of the *Enterobacteriaceae* plus *P. aeruginosa* (8). Among healthy elderly volunteers the rate was 9%, but with ill patients the prevalence increased with increasing severity of illness and level of care up to a rate of 60% (12). In an earlier study, Johanson and associates found only a 2% rate of GNR colonization in healthy adults, but the rates were 16% in moderately ill hospitalized patients and 57% in moribund hospitalized patients (5). The methods used in each of these studies were similar to ours. Our data demonstrate an overall prevalence of GNR of 40% for infants from birth through 6 months of age, using the rate of positivity of all cultures. The prevalence rates for normal infants were clearly higher than those of normal adults; this was not accountable for by differences in methods. The density of colonization of infants may also be higher. In the study of normal subjects by Rosenthal and Tager, most of the selective agar plates had 10 or fewer colonies (8). In our study, only 23% of the five most common species recovered had 10 or fewer colonies on the direct swab, and the majority had a density of colonization that could only be quantitated by using a dilution-plating technique.

The rate of pharyngeal colonization with GNR of hospitalized infants over 1 week of age in our study was 40%. Of these infants over 1 week of age, 93% (27 of 29) were under 2.5 months of age. This prevalence is identical to the prevalence of GNR colonization of normal infants during periods B and C, representing infants 3 days to 2.5 months of age. Thus, while our study shows a high prevalence of infants at risk of nosocomial GNR infection, the fact that this prevalence is similar to that of normal infants at low risk for serious GNR infection suggests that pharyngeal cultures of infants that yield GNR may not correlate with the risk of serious infections. This does not mean that pharyngeal cultures in a neonatal intensive care unit have no use. In the context of nosocomial transmission of organisms of a specific species, organisms with antibiotic resistance, or organisms with a virulence marker, there may be utility in determining pharyngeal colonization rates.

This study demonstrates that there is a substantial prevalence of GNR colonization in normal infants in the first 6 months of life; this prevalence is the highest reported for any other age group of normal subjects. It is not clear where the organisms that colonize the pharynx come from. If colonization was due to acquisition of maternal organisms at the time of birth, the prevalence should have been highest in the youngest age group; however, the prevalence rose during the first 2.5 months of life, suggesting either an exogenous source of bacteria or self-inoculation from a gastrointestinal source. If the hospital was the source of these bacteria, one would expect the same prevalence pattern as with maternal acquisition.

This study was not specifically designed to determine the effects of breast feeding on GNR pharyngeal colonization. The finding that currently breast-fed infants had a significantly lower prevalence of GNR was not expected and should be interpreted cautiously. Relative deficiency of fibronectin may play a role. Woods et al. (13) have shown that fibronectin, a cell surface protein, appears to be important in the prevention of surface colonization with GNR. Levels are lower in seriously ill adults (13) and in infants compared with those in normal adults (3). Lower plasma levels of fibronectin have been found in formula-fed infants than in breast-fed infants (3), and this may explain their higher colonization rate. Other immune factors, such as immunoglobulin A and lymphocytes in breast milk, may be important in determining the different rates of colonization of the infants in this study. It was not possible to compare breast-fed and non-breast-fed infants for all possible confounding variables. For instance, while only 12 of the 49 enrolled subjects came from the suburban middle-class pediatric group, they made up half of the breast-fed infants. Finally, breast-fed infants were nearly as likely as non-breast-fed infants to be colonized with GNR on at least one culture taken during the whole study (64% versus 71%).

The medical implications of pharyngeal colonization with GNR during year 1 of life are unknown. The relationship of GNR colonization and an association of risk of invasive infections has been appreciated in studies of intensive care units. Sprunt et al. (11) have shown that acquisition of a predominant bacterial flora other than alpha-hemolytic streptococci is a risk factor for the development of invasive infection by that strain in the neonatal intensive care unit, but there are no such health implications for normal infants. Thus, these prevalence rates are important for (i) increasing knowledge about normal flora in infants, (ii) providing data for the interpretation of pharyngeal cultures of normal infants that grow GNR, and (iii) providing control data for the study of GNR prevalence in sick or hospitalized newborns.

It appears that the prevalence of pharyngeal colonization with GNR in hospitalized infants is similar to that of age-matched normal controls. Future studies of pharyngeal colonization in sick infants attempting to establish a link between prevalence of GNR and risk of infection must employ comparative data on normal infants.

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