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Research Article

Earliest Culturable Bacteria to Colonize the Intestinal Colon of Neonates Born in Irrua Specialist Teaching Hospital, Irrua, Edo State, Nigeria

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Abstract

The gut microbiome is said to be associated with human health and the understanding of bacteria colonization of the gut ecosystem is crucial to our knowledge on disease development in humans. This study was conducted to establish the earliest bacteria that colonize the intestinal colon of neonates born in Irrua Specialist Teaching Hospital (ISTH) Irrua, Edo State Nigeria and to determine the antibiogram of these isolates. Stool samples were collected at time interval of less than 10 hrs, greater than 10 hrs and after 18 hrs. The samples were processed using standard microbiological methods and Antibiotic susceptibility was done by disc diffusion method. The results revealed that of the 77 isolates, the frequencies of occurrence were *Escherichia coli* 51 (67.5%), *Proteus mirabilis* 13 (16.9%), *Enterobacter* aerogens. 8 (9.1%), *Citrobacter freundii* 3 (3.9%) and *Serratia marcescens 2* (2.6%). *Escherichia coli* was most prevalent accounting for 67.5% of the isolates. The results also showed statistically that comparing the mode of delivery to the mode of nutrition, the mode of delivery ($P<0.05$) determines the colonization pattern of the earliest bacteria in the intestinal colon of neonates but the mode of nutrition ($P>0.05$) does not. The results also show that the meconium is sterile and colonization only begins from 10 hours after birth.%). All isolates from the different neonatal stool samples showed different susceptibility patterns to the antibiotics used. *Escherichia coli* was most resistant to Gentamycin, *Proteus mirabilis* was most resistant to Cloxacillin, *Enterobacter aerogens* and *Citrobacter freundii* to Ofloxacin, and *Serratia marcescens* was most resistant to Ceftazidime.

Keywords

Meconiun, Neonates, Earliest Culturable Bacteria, Intestinal Colon

1. Introduction

The neonatal intestinal microbiota is a complex ecosystem composed of numerous genera, species, and strains of bacteria [1]. This enormous cell mass performs a variety of unique activities that affect both the colonic and systemic physiology.

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Its primary activities include nutritive, metabolic, immunological and protective functions. The intestinal colon of the neonate is generally sterile but within hours of birth, the baby acquires a complex collection of microorganisms, which populate the mouth—then eventually the full length of the track will be colonized. The development of specific microorganisms is influenced by the exposure to certain factors such as maternal microbiota, environmental contact, mode of delivery and the infant's diet [2].

Most studies of infants have been based on faecal samples using the classical plating techniques with culturing on specific media [3]. The establishment of the gut microbial population is not strictly a succession in the ecological sense; it is rather a complex process influenced by microbial and host interactions and by external and internal factors. The climax intestinal flora is attained in successive stages. The foetal intestine is sterile and bathed in swallowed amniotic fluid. Following delivery, multiple different antigens challenge the intestine of the newborn. According to [4], the maternal intestinal flora is a source of bacteria for the neonatal gut. The bacterial flora is usually heterogeneous during the first few days of life, independently of feeding habits. After the first week of life, a stable bacterial flora is usually established. In full-term infants, a diet of breast milk induces the development of a flora rich in *Bifidobacterium* spp [5]. Other obligate anaerobes, such as *Clostridium* spp. and *Bacteroides* spp., are more rarely isolated and also Enterobacteria and Enterococci are relatively few. During the corresponding period, formula-fed babies are often colonized by other anaerobes in addition to Bifidobacteria and by facultative anaerobic bacteria [6]. In another study, the presence of a consistent number of Bifidobacteria in infants delivered in large urban hospitals was not demonstrated, rather the predominant faecal bacteria were coliforms and *Bacteroides*, and according to that study, environmental factors rather than breastfeeding influenced gut colonization after delivery [7]. Environmental factors are indeed extremely important for the intestinal colonization of infants born by caesarean section [8]. In these infants, the establishment of a stable flora characterized by a low incidence of *Bacteroides* spp. and by the isolation of a few other bacteria is consistently delayed. In extremely low-birthweight infants, hospitalization in neonatal intensive care units, characterized by prolonged antibiotic therapy, parenteral nutrition, delayed oral feedings and intubation seems to affect the composition of the intestinal microbiota [9]. According to the few studies so far performed, the predominant species are *Enterococcus faecalis*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* as reported by [10]. Hygienic conditions and antimicrobial procedures strongly influence the intestinal colonization pattern and of all the parts of the digestive tract, the intestines are where the majority of the microflora resides. The microbes can exit the digestive tract through the rectum and the anus via faeces.

The pH of an infant's stomach ranges approximately from 2 to 5. Initially, the pH of the stomach is less acidic, but studies have shown that the presence of microbes, such as *Streptococcus and Lactobacillus*, and their metabolic activities create a more acidic environment. However, further down the digestive tract the acidity of the environment decreases. The infant's stomach is a well-oxygenated area because air swallowed with food arrives in the stomach within moments of ingestion. The facultative anaerobes established in the stomach utilize the available oxygen, resulting in an oxygen-reduced environment for the obligate anaerobic microbes in the intestines [11].

As reported by [12], the mode of delivery determines the nature of microbes ingested by the infant. Through normal vaginal birth, an infant is exposed to the mother's vaginal and faecal flora, which results in the colonization by *Lactobacillus*, *Bifidobacterium*, *Escherichia coli*, and *Enterococcus*. However, an infant delivered by Caesarian section is exposed to a different assortment of microbes, such as *Clostridium* and *Streptococcus*, which are acquired from the tools used. These microbes can establish and colonize rapidly within the sterile digestive tract because there are no pre-existing microbes to compete with [13].

Staphylococcus aureus is normally transferred from the mother's nipple during breastfeeding as well as through mouth contact which is not found in formula-fed infants. The digestive tract of breast-fed infants is colonized by primarily Bifidobacterium while the digestive tract of formula-fed infants is colonized predominantly by Bacteroides with some Bifidobacteria; but over time the difference in the number of colonies of the two genera decreases. Thus it will be necessary to conduct a study of the earliest bacteria flora in the gut of neonates in our study population.

2. Materials and Methods

2.1. Study Site

This study was carried out in Irrua Specialist Teaching Hospital (ISTH). ISTH is a Federal Government of Nigeria Teaching Hospital located in Irrua, Edo State Nigeria. Irrua is administrative headquarters of Esan Central Local Government Area. It is located at Latitude $5^0$15 48^0E. It has a population of about 40,000 people whose major occupation includes Farming, Teaching, Civil services and Trading. The hospital is less than 2km from Ekpoma town and serves as the teaching hoapital to Ambrose Alli University Ekpoma medical programme and it currently has a bed space capacity of three hundred.

2.2. Sample Collection

Following ethical approval from the ethical committee of Irrua specialist teaching Hospital, Irrua and informed consent from mothers, 140 stool samples were collected from 70 neonates born at Irrua Specialist Teaching Hospital Irrua, between February 2017 to April 2018, through the use of sterile rectal Swabs. The meconium (Baby's first stool) were collected

from fresh contaminated diaper by scooping the stool with the sterile swab stick aseptically and taken to the laboratory within a maximum period of 30 mins for analysis. Subsequent stool samples were collected from the neonates after 10 hours and 18 hours by inserting a moistened (Normal Saline) sterile swab stick (Oxoid) into the rectum of the neonates, 1 inch deep and gently rotated. On removal of the swab stick from the anus of the neonates, it was immediately taken to the laboratory for analysis. These stool samples were collected on the average of two from each neonate due to the duration the baby and the mother remained in the hospital after birth.

2.3. Isolation and Identification of Bacteria from Stool Samples

The isolation and identification of bacterial isolates was done following standard microbiological procedures. Stool samples were aseptically smeared on the surfaces of Nutrient agar, Blood agar, and MacConkey agar respectively to form a well of inoculum. The well of inoculum of each plate was then streaked aseptically close to the Bunsen burner, with the use of a sterile wire loop. The plates were well labelled and then incubated at 37^0C for 24 hours. Thereafter, distinct and ambiguous colonies were selected from the overnight cultured plates and subcultured onto Blood Agar and MacConkey agar respectively to obtain pure culture colonies. Identification of bacterial isolates was achieved following Gram staining, and some biochemical tests which included motility, indole, urease, catalase, coagulase, citrate, oxidase, sugar fermentation. Identification of each isolates was confirmed as described by [14]. The identified bacteria were inoculated into nutrient agar slant and stored as stock cultures and kept in the refrigerator at -20^0, properly labelled for further use.

2.4. Antibiotic Susceptibility Test

A 0.5 Macfarland turbidity standard match of each test bacterial isolate was poured on the surface of the Mueller hinton agar plate and was notched round, the excess was discarded into a disinfectant jar and the plates were left to stand on the bench top for 10 minutes. The antibiotic disc, containing some antibiotics commonly used within the study area like augmentin, ofloxacine, cloxacillin, erythromycin, gentamycin, cefuroxime, ceftriazone, was aseptically placed on the surface of the inoculated plates and incubated at 37°C for 24 hours, after which the zone of inhibitions was recorded in mm and within the limits published by [15].

3. Results

In the screening of 140 stool samples from 70 neonates born at Irrua Specialist Teaching Hospital, Irrua, Edo State, a total of 77 Gram-negative facultative anaerobes were obtained following biochemical identification. The bacterial isolates included *Escherichia coli, Proteus mirabilis, Enterobacter* aerogens, *Citrobacter freundii,* and *Serratia marcescens*. Of the 77 isolates that were recovered, 51 (67.5%) were *Escherichia coli*, 13 (16.9%) were *Proteus mirabilis*, 8 (9.1%) were *Enterobacter* aerogens, 3 (3.9%) were *Citrobacter freundii*, and 2 (2.6%) were *Serratia marcescens* (Table 1). *Escherichia coli* was the only bacteria isolated from meconium that was only collected after 10 hours is likely not to be sterile. Table 2 shows the distribution of bacterial isolates according to mode of delivery and nutrition, where it was shown that from the total 51 *Escherichia coli* isolated in this study, 24 (47%) were recovered from neonates born through vaginal delivery while 27 (53%) were recovered from neonates born through cesarean delivery and based on their mode of nutrition, 39 (76%) of the *Escherichia coli* isolated were recovered from neonates strictly feeding on breast milk while 12 (24%) were gotten from neonates feeding on mixed formula. For the 13 *Proteus mirabilis* isolates, in terms of mode of delivery, 10 (77%) were recovered from vaginal delivery while 3 (23%) were recovered from cesarean delivery. For mode of nutrition, 8 (62%) were isolated from neonates feeding on breast milk only while 5 (38%) were recovered from neonates feeding on mixed formula. Analysis of all these bacterial isolates revealed that there was a significant effect on the colonization pattern of earliest bacteria in the intestinal colon of neonates by their mode of delivery ($P<0.05$) but no significant effect of nutrition on the colonization of bacteria at this stage of life ($P>0.05$).

Table 1. *Bacterial Isolates from the Meconium and Stool Samples of Neonates.*

Bacterial Isolates	No of Isolates from: Meconium Second <10 >10hrs Stool>18hrs	Total (%)
Escherichia coli	- 4 47	51 (67.5)
Proteus mirabilis	- - 13	13 (16.9)
Enterobacter aerogenes	- - 8	8 (9.1)
Citrobacter freundii	- - 3	3 (3.0)
Serratia marcecens	- - 2	2 (2.6)

Bacterial Isolates	No of Isolates from: Meconium Second <10 >10hrs Stool>18hrs	Total (%)
% Distribution of isolates	- (0%) 4 (5%) 73 (95%)	77 (100%)

Table 2. *Distribution of Bacterial Isolates according to Mode of Delivery and Nutrition.*

	Mode of Delivery a	Mode of Nutrition b
Bacteria Isolates/Number	SVD (35) CS (35)	Breast Mixed
Escherichia coli 51	24 27	39 12
Proteus mirabilis 13	10 3	8 5
Enterobacter aerogenes 8	6 2	7 1
Citrobacter freundii 3	2 1	2 1
Serratia marcescens 2	1 1	2
Total 77	43 34	58 19
Mean	8.6 6.8	11.6 3.6

KEY
SVD = Sponteneous Vaginal Delivery
CS = Cesarean Section
Mixed = Both breast and formula fed
Using t-test of significant difference at 95% confidence limit (P<0.05)
a: t = 2.86
b: t = 4.32
a: t (P< 0.05)
b: t (P>0.05)

Antibiogram of Bacterial Isolates

Antibiotic susceptibility results carried out on the bacterial isolates shows that *Escherichia coli* were most resistant to Gentamicin and least resistant to Ofloxacin. Of the 51 *Escherichia coli* isolated, 28 (54%) were resistant to Gentamicin while 15 (17%) were resistant to Ofloxacin. *Proteus mirabilis* was most resistant to Cloxacillin 9 (69%) and least resistant to Gentamicin 3 (23%). A total of 6 (75%) out of 8 isolates of *Enterobacter aerogenes* were resistant to Ofloxacin, while 2 (67%) of the 3 *Citrobacter freundii* were resistant to both Ofloxacin and Ceftazidime and *Serratia marcescens* (50%) were resistant to Ceftazidime (Table 3). From the result obtained as shown in Table 3, it can be seen that the degree of antibiotic-resistance for some bacteria isolates was very high and ranged between 45 - 51%. The results also revealed that *Escherichia coli*, *Proteus mirabilis* and *Enterobacter* sp were multidrug resistant because they were resistant to more than 3 of the 8 (above 37%) antibiotics used in this study while *Citrobacter freundii* and *Searratia marcesens* where not multidrug resistant.

Table 3. *Antibiotic Susceptibility Profile of Bacterial Isolate from Stool Samples of Neonates.*

Bacterial Isolates Con/disc (mcg)	Ceftazidime 30	Cefurox-ime 30	Gen-tamicin 10	Ceftriax-one 30	Erythro-mycin 5	Cloxa-cillin 5	Oflox-acin 5	Aug-mentin 30	Total	MDR (%)
Escherichia coli (51)	S (23)	(27)	(21)	(25)	(32)	(22)	(19)	(29)	S (198)	MDR (45%)
	I (6)	(4)	(2)	(0)	(1)	(4)	(7)	(2)	I (26)	
	R (22)	(20)	(28)	(26)	(18)	(15)	(15)	(20)	R (164)	

Bacterial Isolates Con/disc (mcg)	Ceftazidime 30	Cefurox-ime 30	Gen-tamicin 10	Ceftriax-one 30	Erythro-mycin 5	Cloxa-cillin 5	Oflox-acin 5	Aug-mentin 30	Total	MDR (%)
Proteus mirabilis (13)	S (6)	(6)	(9)	(8)	(5)	(3)	(6)	(5)	S (48)	MDR (47%)
	I (0)	(0)	(1)	(0)	(2)	(1)	(1)	(0)	I (5)	
	R (7)	(7)	(3)	(5)	(6)	(9)	(6)	(8)	R (44)	
Enterobacter sp (8)	S (4)	(5)	(3)	(4)	(2)	(4)	(2)	(4)	S (28)	MDR (51%)
	I (0)	(1)	(0)	(2)	(1)	(1)	(0)	(2)	I (7)	
	R (4)	(2)	(5)	(2)	(5)	(3)	(6)	(2)	R (29)	
Citrobacter freun-dii (3)	S (1)	(3)	(2)	(1)	(2)	(2)	(1)	(2)	S (14)	NMDR (33%)
	I (0)	(0)	(0)	(1)	(1)	(0)	(0)	(1)	I (3)	
	R (2)	(0)	(1)	(1)	(0)	(1)	(2)	(0)	R (7)	
Serratia marcesens (2)	S (1)	(1)	(2)	(2)	(1)	(1)	(2)	(2)	S (12)	NMDR (20%)
	I (0)	(0)	(0)	(0)	(0)	(1)	(0)	(0)	I (1)	
	R (1)	(1)	(0)	(0)	(1)	(0)	(0)	(0)	R (3)	

Keys:
S - Sensitive (≥ 17mm)
I - Intermediate (14mm-16mm)
R - Resistant (≤ 13mm)
MDR - Multidrug resistant
NMDR - Non multidrug resistant

4. Discussion

The result of the incidence of bacteria isolated from 140 neonatal stool samples obtained from Irrua Specialist Teaching Hospital, Irrua, Edo State, Nigeria revealed the isolation of *Escherichia* coli, *Proteus mirabilis*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Serratia marcescens*. It showed *Escherichia coli* as the most prevalent earliest culturable bacteria to colonize the intestinal colon of neonates between 1 to 4 days old. This is in line with the report of [16] that reported *Escherichia coli* as the most prevalent bacteria to first colonize the gastrointestinal colon of neonates.

The 5 bacterial isolates obtained in this study were also found to be Gram-negative facultative anaerobes in line with the report of [2] which demonstrated that upon delivery, the neonatal gastro intestinal tract (GIT) is an aerobic environment hence, it must be converted into an anaerobic environment for the anaerobic bacteria to colonize, preparation to the second phase of colonization. This shift from the aerobic environment of the neonatal GIT to the anaerobic environment can only be actualized by the help of facultative anaerobes hence, the first phase of neonatal colonization should be facultative anaerobes, as we have demonstrated from this study.

It was also shown from the distribution of bacterial isolates that the colonization pattern of the earliest (First phase) bacteria of the neonatal GIT is not a function of the mode of feeding (either breast milk or mixed feeding) at this stage of the neonate. This is in conformation with the finding of [6] that the first phase of colonization, bacteria colonize the Gastrointestinal colon of neonates irrespective of the pattern of feeding and that the pattern or mode of feeding only influences the colonization pattern of the second phase, which comprises anaerobes.

Considering the effect of mode of birth with respect to the earliest bacteria to colonize the intestinal colon of neonates, from the result obtained in this study, the mode of birth either through normal vaginal delivery or cesarean section has an effect on the pattern of earliest bacteria to colonize the intestinal colon of neonates born in ISTH. This fact can be tied to the findings of [11] which stated that an ideal infant colonization begins with intrinsic (oral inoculation of maternal vaginal microbiota and maternal intestinal bacteria) for babies delivered through the vagina and then extrinsic (environmental) inoculation.

From the result of this study, *Escherichia coli* was more prevalent from neonates born through cesarean section than those born through normal delivery but there is a clear dichotomy in number of *Enterobacter* aerogenes, *Proteus mirabilis* and *Citrobacter freundii* which is higher in neonates born through normal delivery than those born through cesarean delivery because these bacteria are normal flora of the maternal

gastro intestinal tract which can easily be swallowed up during birth for vaginal delivered neonates from feces or vaginal fluid, as opposed to neonates born by cesarean section. This correlates with findings of [8], where he demonstrated that bacteria isolates from stool samples of neonates born through vaginal delivery reflect the normal flora of their mother's intestinal microbiota while those born through cesarean delivery instead reflects the microbes present in the delivery environment.

It was also found from this study that meconium is sterile. This is due to the fact that meconium of neonates collected immediately after birth or less than 4 hours after delivery, yielded no growth of micro-organisms but 80% of samples collected after many hours of birth yielded growth of bacteria either because the neonates have commenced feeding or nosocomial inoculation must have occurred. This is in contrast with the result of [17] who quoted that bacteria have been isolated from meconium, umbilical cord blood, and amniotic fluid. It is our opinion that the report of [17] is indicative of a systemic infection acquired from the mother.

Antibacterial susceptibility result of the bacterial isolates obtained in this study showed sensitivity and resistant to commonly used antibiotics which presupposes that the bacterial isolated from this study did not appear spontaneously. The resistant of the isolates is a result of acquired resistant bacteria from the mother or the environment. This study reveals that *Escherichia coli* were resistant 54% and 17% for Gentamicine (54%) and Ofloxacine (17%). *Enterobacter* species was mostly resistant to Ofloxacine 75%. This findings slightly contradicts that of [18] where most of the bacterial isolates were sensitive to most commonly used antibiotics in Bessarabia.

This study did not look at strain relatedness from bacteria isolated from both mother and the new born infant which could have explain if the resistant isolates recorded from the babies correlated with that of the mothers.

From the results obtained, it was also shown that the degree of antibiotic-resistant bacteria isolates was very high which should be of great concern to neonates born in Irrua Specialist Teaching Hospital especially to commonly used antibiotics such as Ofloxacin, Cloxacillin and Ceftazidime to which the bacterial isolates show a high level of resistant. However, most of the bacterial isolates were sensitive to antibiotics such as Erythromycin and Augmentin.

5. Conclusion

Stool samples analyzed to determine the earliest culturable bacteria that first colonize the intestinal colon of neonates revealed the presence of *Escherichia coli*, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter aerogenes* and *Citrobacter freundii*. These bacterial isolates were found to be Gram-negative facultative anaerobes, which were the earliest bacteria to colonize the intestinal colon of neonates and set the stage for subsequent colonization by possibly anaerobes through the conversion of the aerobic environment of the GIT to an anaerobic environment. The colonization of these earliest bacteria is determined by the mode of delivery either vaginal delivery or cesarean section while the mode of feeding (Breast-feeding or mixed formula) does not affect the colonization of these earliest bacteria. The result of this study has also revealed that meconium is sterile and that colonization only begins a few hours post to delivery.

Abbreviations

ISTH	Irrua Specialist Teaching Hospital
CLSI	Clinical and Laboratory Standard Institutes
SVD	Spontaneous Vaginal Delivery
CS	Cesarean Section

Author Contributions

Esumeh Frederich Ikechukwu: Project administration, Supervision, Validation

Igbinosa Noel: Formal Analysis, Investigation, Resources, Writing – original draft, Writing – review & editing

Inyang Nyoho Johnson: Resource Visualization

Obiazi Helen Akinagu: Conceptualization

Ofanson Julius Ehikioya: Resources, Visualization

Ikuejamofo Seun Augustine: Visualization, Resource

Data Availability Statement

The data used in this article are sourced from materials mentioned in the references.

Conflicts of Interest

The authors declare no conflict of interest.

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