Study of Earliest Bacteria to Colonize the Intestinal Colon of Neonates in Irrua Specialist Teaching Hospital, Irrua, Nigeria

[1]Igbinosa N., [2]Aliemhe C., [1]Ochakpor, D.O. and [3]Ebhomen S. E.
[1]Department of Microbiology, Faculty of Life Sciences, Ambrose Alli University Ekpoma, Nigeria.
[2]Biological Sciences of Laboratory Technology Department, School of Applied Sciences, Auchi Polytechnic, Auchi, Nigeria.
[3]Department of Medical Microbiology, Faculty of Medical Laboratory Science, Ambrose Alli University Ekpoma, Nigeria

ABSTRACT
Background; *The intestinal colon of the neonate is generally sterile but within hours of birth, the baby acquires a complex collection of microorganisms.*

Aim; *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.*

Method*; Stool samples were collected at time interval of less than 10hrs, greater than 10hrs and after 18hrs.The samples were processed using standard microbiological methods and Antibiotic susceptibility was done by disc diffusion method.*

Result; *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%). Escherichiacoli 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.*

Conclusion; *The study shows that meconium is sterile and colonization only begins from 10 hours after birth. All isolates from the different neonates 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.*

INTRODUCTION

The neonatal intestinal microbiota is a complex ecosystem composed of numerous genera, species, and strains of bacteria (Palmer *et al.*, 2007). This enormous cell mass performs a variety of unique activities that affect both the colonic and systemic

physiology. 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 (Azad *et al.*, 2013).

Most studies of infants have been based on faecal samples using the classical plating techniques with culturing on specific media (Fanaro *et al.*, 2003). 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 new-born. According to Makino *et al.,* (2013), 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* (Mountzouris *et al.,* 2002). 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 (Azad et al., 2013). 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 (Haarman *et al.,* 2006). Environmental factors are indeed extremely important for the intestinal colonization of infants born by caesarean section (Haarman *et al.*,2006). 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 (Isolauri *et al.*,1995). 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 Haarman *et al.,* (2006). Hygienic conditions and antimicrobial procedures strongly influence the intestinal colonization pattern.

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 (Berseth *et al.,* 2006). 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 (Penders *et al.,* 2006; Parracho *et al.*,2007).

As reported by to Makino et al., (2013), 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 Caesarean 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 (Favier *et al.,* 2002; Favier *et al.,* 2003).

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 *Bifidobacteria* 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

MATERIALS AND METHODS

Sample Collection

Following ethical approval from the ethical committee of Irrua specialist teaching Hospital, Irrua and informed consent from the babies' mothers, 140 stool samples were collected from 70 neonates at Irrua Specialist Teaching Hospital, Irrua 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 30mins for analysis. Subsequent stool samples were collected from the neonates after 30-48 hours by inserting a moistened (Normal Saline) Sterile Swab stick 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.

Isolation of Bacteria from Stool Samples.

Stool contaminated swab sticks 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 purified or subcultured onto Blood Agar and MacConkey Agar respectively to obtain pure culture colonies. The identified organisms were inoculated into nutrient agar slant and stored as stock cultures and kept in the refrigerator at -20^0, properly labelled for further use.

Antibiotic Susceptibility Test

A 0.5 Macfarland turbidity standard (Clinical and Laboratory Standards Institution, 2008) 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 10minutes. The antibiotic disc was placed on the surface of the inoculated plates and incubated at 37^oC for 24 hours, after which the zone of inhibitions was recorded in mm and within the limits published by CLSI (2008).

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 were the only bacteria isolated from meconium that was only collected after 10 hours which is likely not to be sterile.

Table 2 shows the distribution of bacterial isolates according to delivery and nutrition.

There was a significant effect on the colonization pattern of earliest bacteria in the intestinal colon of subjects by mode of delivery ($P<0.05$) but not by nutrition ($P>0.05$).

Table 1: Bacterial Isolates from the Meconium and Stool Sample of Neonates

Bacteria Isolates	No of Isolates from: Meconium <10	>10hrs	Second Stool	Total(%)
Escherichia coli	-	4		51(67.5)
Proteus mirabilis	-	-		13(16.9)
Enterobacter aerogenes	-	-	8	8(9.1)
Citrobacter freundii	-	-		3(3.0)
Serratiamarcecens	-	-		2(2.6)
% Distribution of isolates		4(5%)	73(95%)	77(100%)

Table 2: Distribution of Bacterial Isolates According To Mode of Delivery and Nutrition.

Bacteria Isolates/Number	Mode of Delivery [a] SVD(35)	CS(35)	Mode of Nutrition [b] BREAST	MIXED
Escherichia coli 51	24	27	39	12
Proteus mirabilis 13	10	3	8	5
*Enterobacter*aerogenes8	6	2	7	1
*Citrobacterfreundii*3	2	1	2	1
*Serratiamarcescens*2	1	1	2	
Total 77	43	34	58	19
Mean	8.6	6.8	11.6	3.6

KEY
SVD = Spontaneous Vaginal Delivery
CS = Caesarean 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* was resistant (50%) to Ceftazidime. From the result, the degree of antibiotic-resistant bacteria isolates was very high and ranged between 50 – 75%.

TABLE 3: Antibiotic Susceptibility Profile of Bacterial Isolate from Stool Samples of Neonates

SUSCEPTIBILITY OF BACTERIAL ISOLATES TO:

BACTERIAL ISOLATE		Ceftazidime	Cefuroxime	Gentamicin	Ceftriaxone	Erythromycin	Cloxacillin	Ofloxacin	Augmentin	Total
	Con/disc (mcg)	30	30	10	30	5	5	5	30	
Escherichia coli (51)	S	(23)	(27)	(21)	(25)	(32)	(22)	(19)	(29)	S(198)
	I	(6)	(4)	(2)	(0)	(1)	(4)	(7)	(2)	I (26)
	R	(22)	(20)	(28)	(26)	(18)	(15)	(15)	(20)	R(164)
Proteus mirabilis (13)	S	(6)	(6)	(9)	(8)	(5)	(3)	(6)	(5)	S(48)
	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)
	I	(0)	(1)	(0)	(2)	(1)	(1)	(0)	(2)	I (7)
	R	(4)	(2)	(5)	(2)	(5)	(3)	(6)	(2)	R(29)
Citrobacterfreundii (3)	S	(1)	(3)	(2)	(1)	(2)	(2)	(1)	(2)	S(14)
	I	(0)	(0)	(0)	(1)	(1)	(0)	(0)	(1)	I (3)
	R	(2)	(0)	(1)	(1)	(0)	(1)	(2)	(0)	R(7)
Serratiamarcesens (2)	S	(1)	(1)	(2)	(2)	(1)	(1)	(2)	(2)	S(12)
	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)**

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 bacteria to colonize the intestinal colon of neonates between 1 to 4 days old. This is in line with the report of Dominguez-Bello *et al.*, (2010) 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 which agrees with the report of Johnson and Versalovic, (2012) which demonstrated that upon delivery, the neonatal 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 (Table 4) that the colonization pattern of the earliest (First phase) colonizing 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 Mander and Mikelsaar, (1996) 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 delivery or caesarean 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 Makino *et al.*, (2013) which states 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 caesarean 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 caesarean 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 faeces or vaginal fluid but cannot be swallowed by caesarean born neonates. This correlates with findings of Dominquez-Bello *et al* (2010), 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 caesarean 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 10hours prior to delivery, yielded no growth of micro-organisms but 80% passed and 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 Funkhouser and Bordenstein, (2013) who quoted that bacteria have been isolated from Meconium, umbilical cord blood, and Amniotic fluid. It is our opinion that the report of Funkhouser and Bordenstein (2013) is indicative of a systemic infection acquired from the mother.

Antimicrobial resistant is a serious global issue resulting in death, but the new-borns babies are particularly at risk because of immature immunity, this may be as a result of exposure in neonatal intensive care unit, part of the problem may be due to contaminated environment and equipment used. A study in India showed an increase of 2 to 52% from 2002 to 2009 respectively and the organisms involved ranged from E. coli, *Kelbsiella*, Pseudomonas and Acinetobacter which accounted for 53% of infection in new-born ward (http:longitude prize.org). Studies have been conducted in different setting and most of the microorganisms incriminated in neonatal sepsis are well documented and the challenges of multidrug resistance in treatment is a global issue, but studies have not been able to link if neonatal sepsis could be caused by the early colonizers considering the neonate immune status in early life. This study isolated *Escherichia* coli, *Proteus mirabilis, Enterobacter* aerogenes, *Citrobacter freundii* and *Serratia marcescens* as the early organisms isolated from the neonates.

Antibacterial susceptibility result of the bacterial isolate collected in the study showed multi drug resistance to the antibiotics used which presupposes that the microorganism isolated in the study did not appear spontaneously this is also evident in the fact that there was a significant difference between feeding of the babies and microbial colonization. The resistance of the isolates is a result of acquired resistance from the mother. This study reveals that Escherichia coli were resistant for Gentamicin and Ofloxacin. Enterobacter species was mostly resistant to Ofloxacin (75%). 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 resistance recorded from the babies correlated with that of the mothers.

Conclusion

Stool samples analysed to determine the earliest bacteria that first colonize the intestinal colon of neonates revealed the presence of *Escherichia coli, Proteus mirabilis, Serratia marcescens, Enterobacter* spp 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 caesarean section while the mode of feeding (Breastfeeding 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 prior to delivery.

REFERENCES

Azad, M.B., Konya, T., andMaughan, H. (2013). Gut *microbiota* of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *Canadian Medical Association Journal.***185***(5)*:385–394.

Berseth, C.L., Thureen, P.J., and Hay, W.W. (2006).Development of the gastrointestinal tract.*Neonatal Nutrition and Metabolism.2*:67-73.

Chessbroug, M. (2006). District Laboratory Practice in tropical countries: *In Microbiological Tests*. Second Edition. Pp 62-70

Clinical and Laboratory Standards Institute (CLSI) (2008). Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement. CLSI document M100-18. Wayne, PA: Clinical and Laboratory Standards Institute.

Dominguez-Bello, M.G., Costello, E.K. and Contreras, M. (2010). Delivery mode shapes the acquisition and structure of the initial *microbiota* across multiple body habitats in newborns. *Proceedings National Academic Science.***107**(26):11971–11975.

Fanaro, S., Chierici, R., Guerrini, P., and Vigi, V. (2003). Intestinal *microflora* in early infancy: composition and development. *ActaPaediatrica Supplement*. **441**:48-55

Favier, C.F., De Vos, W.M., and Akkermans, A.D. (2003).Development of bacterial and Bifidobacteri*al* communities in feces of newborn babies.*Anaerobe*, **9**:219-229.

Favier, C.F., Vaughan, E.E., De Vos, W.M., and Akkermans, A.D. (2002).Molecular monitoring of succession of bacterial communities in human neonates.*Applied and Environmental Microbiology*, **68**:219-226.

Funkhouser, L.J. and Bordenstein, S.R.(2013). Mom Knows Best: The Universality of Maternal *Microbial* Transmission. *PLoS Biology.***11**(8):e1001631.

Haarman, M. and Knol, J. (2006).Quantitative Real-Time PCR Analysis of Fecal *Lactobacillus* Species in Infants Receiving a Prebiotic Infant Formula.*Applied and Environmental Microbiology*, **72**:2359-2365.

Isolauri, E., Joensuu, J., *Suomalainen*, H., Luomala, M., and Vesikari, T. *(*1995). Improved immunogenicity of oral D 3RRV reassortant rotavirus vaccine by *Lactobacillus*casei GG.*Vaccine*, **13**:310-312.

Johnson, C.L. and Versalovic, J. (2012).The human *microbiome* and its potential importance to pediatrics.*Pediatrics*.**129**(5):950–960.

Makino, H., Kushiro, A. and Ishikawa, E. (2013). Mother-to-infant transmission of intestinal Bifidobacteri*al* strains has an impact on the early development of vaginally delivered infant's *microbiota*. *PLoS One.***8**(11):e78331.

Mountzouris, K., McCartney, A., and Gibson, G. (2002).Intestinal *microflora* of human infants and current trends for its nutritional modulation.*British Journal of Nutrition*, 87:405-420.

Neu, J., Douglas-Escobar, M., and Lopez M. (2007).Microbes and the Developing Gastrointestinal Tract.*Nutrition in Clinical Practice*, **22**:174-182.

Palmer, C., Bik, E.M., DiGiulio, D.B., Relma, D.A., and Brown, P.O. (2007).Development of the Human Infant Intestinal Microbiota.*PLoS Biology*,**5**:1556-1568.

Parracho, H., McCartney, A., and Gibson, G. (2007).Probiotics and prebiotics in infant nutrition.*Proceedings of the Nutrition Society*, **66**:405–411.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den Brandt, P A., and Stobberingh, E. E. (2006). Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy.*Pediatrics*, **118**:511-521.