Characterization of aerobic Gram-negative gut microflora of mother-baby pairs in normal and caesarean section deliveries

A Thesis submitted to

Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

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Date: March, 2024

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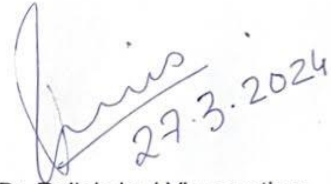
From June 2023 to May 2024

Certificate

This is to certify that this dissertation entitled '**Characterization of aerobic gram-negative gut microflora of mother-baby pairs in normal and caesarean section deliveries**' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work was carried out by **Shikhar Kumar** at **ICMR-National Institute of Virology, Pune** under the supervision of **Dr. Rajlakshmi Viswanathan, Scientist 'E'& Group Leader, Bacteriology** , during the academic year 2023-2024.



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Declaration

I hereby declare that the matter embodied in the report entitled '**Characterization of aerobic gram-negative gut microflora of mother-baby pairs in normal and caesarean section deliveries**' are the results of the work carried out by me at the Bacteriology Group, **ICMR-National Institute of Virology, Pashan, Pune**, under the supervision of **Dr. Rajlakshmi Viswanathan** and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Shikhar Kumar

Date: 27-03-2024

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Acknowledgments

I would like to use this opportunity to offer my heartfelt gratitude and appreciation to everyone who helped and contributed to the completion of my MS thesis, " Characterization of aerobic Gram-negative gut microflora of mother-baby pairs in normal and caesarean section deliveries." This research journey has been an important period in my academic and personal development, and I am deeply grateful for the advice, encouragement, and help I have received.

First, I want to express my heartfelt gratitude to my supervisor, Dr. Rajlakshmi Viswanathan. Your experience, patience, and dedication to my research have been priceless. Your advice and constructive feedback have influenced the direction of my work and motivated me to obtain the best results possible. I am appreciative for the opportunity you have given me and for your continuous support throughout this journey.

I want to express my sincere thanks to Dr. Arun, Ms. Sharon, Ms. Shruti, Ms. Meera, Ms. Soundarya and other Bacteriology Group members who generously donated their time and knowledge, without which this research would not have been achievable. Their inputs have significantly increased the value of my research. Your encouragements were critical in the successful completion of my MSc thesis.

I would also like to thank ICMR- National Institute of Virology, Pune for their amazing support and resources during this thesis process, Dr. Gayathri Pananghat for being my expert member and IISER, Pune for providing me with the opportunity for pursuing my master's thesis. At last, I want to thank my friends and family members for their immense support throughout this journey.

Thank you again for your unwavering support.

Abstract

Background

The human gut microbiota plays a crucial role in maintaining host health, and various factors, including mode of delivery at birth influence its composition. This study aimed to comprehensively characterize the cultivable aerobic gram-negative gut microflora in mother-baby pairs born through vaginal and cesarean section (C-section) deliveries, with a focus on understanding the impact of delivery mode on neonatal gut microbiota.

Methods

The study involved processing fecal specimens obtained from mother-baby pairs. Sample verification and documentation were performed. The samples were processed by plating them on MacConkey and ChromAgar plates. Bacterial colonies were identified by Gram staining, hanging drop motility assay, and biochemical tests along with automated identification for few of the unidentified organisms. End point PCR were performed for the detection of antibiotic resistant genes in all the isolates.

Results

Thirty mother-baby pairs (15 each from C-section and vaginal delivery) and their baby's follow-up (10 C-section, 5 vaginal) specimens were screened for cultivable aerobic Gram-negative bacteria isolation and identification. A total of 47 E. coli and 29 K. pneumoniae isolates were obtained. Seven of 32 mother's E. coli isolates showed at least one antibiotic resistant gene (ARG) while 10 had multiple ARGs. Eleven of 16 mother's K. pneumoniae isolates, showed at least one ARG while only one of them had multiple ARGs. Two of 15 baby's E. coli isolates showed at least one ARG while none of them had multiple ARG. 4 of 13 baby's K. pneumoniae isolates showed at least one ARG while 5 of them had multiple ARGs.

Conclusion

In conclusion, the results from this study shed light on the presence, distribution, and characteristics of aerobic Gram-negative bacteria, particularly E. coli and K. pneumoniae, in fecal specimens of mother-baby pairs. The study demonstrated that both C-section and vaginal delivery cases harbour these bacteria, with E. coli being more in number. However, K. pneumoniae showed a higher occurrence in C-section cases. Genotypic assays revealed that the C-section group exhibited a higher number of ARG associated with beta-lactam antibiotics as compared to the vaginal delivery group.

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Chapter 1 Introduction

The human gastrointestinal tract, a dynamic ecosystem, serves as a host to trillions of microorganisms collectively known as the gut microbiota (Colella et al., 2023). This intricate microbial community plays a pivotal role in maintaining host health by contributing to digestion, nutrient absorption, and modulation of the immune system (Brown et al., 2013). At birth, a crucial event unfolds as the neonate is exposed to its first microbial colonizers, marking the initiation of a lifelong relationship between the host and its microbiota (Gritz & Bhandari, 2015). The mode of delivery has been identified as a critical factor influencing the initial composition of the infant gut microbiome (Mitchell et al., 2020). In recent decades, the rising rate of cesarean section (C-section) deliveries has raised concerns regarding potential alterations in the early gut microbiota development (Ríos-Covián et al., 2021). In India, the number of C-section births is increasing. The rate of C-section births in India is 21.5%, according to the National Family Health Survey (NFHS) (Mohan et al., 2023). Following microbial colonization at birth, the gut microbiome is crucial for maintaining the healthy growth of human neonates and has an impact on both health and disease in later life (Sarkar et al., 2021). The initial colonization of the gut begins during delivery, where the newborn is exposed to the maternal microbiota (Kalbermatter et al., 2021). Vaginal delivery exposes the newborn to the maternal vaginal and fecal microbiota, leading to the acquisition of a diverse bacterial community (Rasmussen et al., 2020). In contrast, C-section delivery bypasses the vaginal canal, and the neonate is exposed to a different microbial environment, primarily composed of skin and environmental bacteria (Inchingolo et al., 2024). This disparity in microbial exposure at birth has been shown to influence the composition and diversity of the gut microbiota in early life, with potential implications for the development of the infant's immune system and long-term health outcomes (Dogra et al., 2021). To better understand the vertical and horizontal transfer of microbial flora from the mother to the neonate, it is important to examine the gut flora of mothers and their neonates delivered by vaginal and C-section delivery (Domínguez-Bello et al., 2010). Comparison of the antibiotic resistance patterns of indicator bacteria in neonates delivered by the two different

methods will help to understand the extent of colonization with resistant microflora in the study population (Klassert et al., 2020).

1.1 Background:

The human gut microbiota represents a complex and dynamic community of microorganisms that coexist within the gastrointestinal tract (Dieterich et al., 2018). Comprising bacteria, viruses, fungi, and archaea, the gut microbiota forms a symbiotic relationship with the host, exerting profound effects on various aspects of human health (Matijašić et al., 2020). Among these microorganisms, bacteria are predominant, with an estimated 100 trillion bacterial cells residing in the human gut (Ramakrishna, 2013). This vast microbial community contributes significantly to the metabolism of nutrients, maintenance of gut barrier function, and modulation of the immune system. The neonatal gut is an aerobic environment in which only facultative anaerobes such as Enterobacteriaceae may flourish (Henderickx et al., 2019). The mode of delivery has been identified as a key determinant shaping the initial composition of the infant gut microbiome (Mitchell et al., 2020). Vaginally delivered infants receive a primary inoculum of maternal vaginal and fecal microflora, fostering the establishment of a diverse microbial community (Van Best et al., 2022). In contrast, infants born via Cesarean section, bypassing the birth canal, are exposed primarily to microbes from the mother's skin and the surrounding environment, resulting in a distinctive microbial profile (Inchingolo et al., 2024).

1.2 Aerobic Gram-Negative Bacteria:

Within the vast landscape of the gut microbiota, aerobic Gram-negative bacteria constitute a significant and distinctive subgroup. These bacteria have a unique cell wall structure with lipopolysaccharides in the outer membrane. They play important roles in nutrient cycling, immune modulation, and gut homeostasis. Prominent genera within this group include *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter* which belong to the family Enterobacteriaceae (Milani et al., 2017). Other important Gram-negative bacteria include Pseudomonas and Acinetobacter. Gram negative bacteria exhibit a wide range of functions, from essential commensals contributing to gut health to potential pathogens associated with opportunistic infections (Dey & Chaudhuri, 2022).

Aerobic Gram-negative bacteria in the gut aid in important physiological processes such as breaking down complex dietary carbohydrates, producing essential vitamins, and stimulating the host immune system (Passalacqua et al., 2016). Furthermore, certain pathogenic strains within this group have been linked to gastrointestinal infections, emphasizing the need for a comprehensive understanding of their prevalence and role in the gut of infant (Milani et al., 2017).

1.3 Rationale for the Study:

Gut microbial flora is influenced by several factors including geography, birth term, mode of delivery, nutritional status, and exposure to medications (Kumbhare et al., 2019). There is a lack of understanding regarding aerobic Gram-negative bacteria, particularly between vaginal and C-section deliveries, despite extensive research on the gut microbiota (Coscia et al., 2021). This is particularly true in the Indian context. The national rise in C section rates calls for a more nuanced investigation into the consequences of altered microbial colonization in infants born through C-section (Theophilus & Taft, 2023).

This study aims to address this gap in knowledge by comprehensively characterizing the cultivable aerobic Gram-negative gut microflora in mother-baby pairs. The cultivable flora of newborns born through normal vaginal deliveries will be compared with those born via C-section. This will help to understand colonization patterns in these two groups.

1.5 Significance of the Study:

This study holds significant implications for both maternal and neonatal healthcare. The findings from this study will provide valuable insights into the impact of C-section deliveries on the aerobic gram-negative gut microflora in mother-baby pairs. Understanding the alterations in the microbial composition and potential functional implications associated with C-section delivery may have significant implications for neonatal health. Moreover, these findings could aid in the development of targeted interventions to modulate the gut microbiota in infants delivered via C-section. This would help promote healthy microbial colonization and minimize potential health risks.

In conclusion, this thesis will shed light on the characterization of aerobic gram-negative gut microflora in mother-baby pairs, comparing vaginal and C-section deliveries. By elucidating the impact of delivery mode on the neonatal gut microbiota, this research will contribute to our understanding of early-life microbial colonization and its potential implications for human health. Interventions targeted at optimizing the development of gut microbiota in infants born via C-section are likely to promote their long-term health with certainty.

1.6 Structure of the Thesis:

This thesis is organised into several chapters, each dedicated to specific aspects of the research. Chapter 2 will detail the research methodology, outlining the procedures for sample collection, processing, and analysis. Chapter 3 will present the results of the study, while Chapter 4 will discuss the implications of the findings and their relevance to maternal and neonatal health. The thesis will culminate in Chapter 5, which will offer conclusions, recommendations, and potential avenues for future research. In summary, this thesis aims to unravel the intricate dynamics of aerobic gram-negative gut microflora in mother-baby pairs born through different delivery modes.

Chapter 2 Materials and Methods

2.1. Materials Used

2.1.1. Clinical Specimen

Fecal specimens were collected from healthy individuals (mother-baby pairs), their socio-demographic data along with a consent for this study was taken in accordance with ICMR-NIV institutional scientific and ethics committees.

2.1.2. Culture Media Used

- **Cary Blair Transport Media** (Manufacturer: HIMEDIA, Catalog no. MS202-50NO) was used to inoculate the fecal samples and was transported in a cold chain to the laboratory.
- **MacConkey's Agar without CV, NaCl and with 0.5%Sodium Taurocholate** (Manufacturer: Difco, Catalog no. 247010) was used to differentiate between Lactose Fermenters (LF) and Non-lactose Fermenters (NLF) from the fecal specimens.
- **Coliform ChromSelect Agar** (Manufacturer: Merck, Catalog no. 81938-500GF) was used to differentiate between *Escherichia coli* and *Klebsiella pneumoniae* based on the colony color.
- **Nutrient Agar NA** (Manufacturer: BD, Catalog no. 211795) was used for culturing bacteria.
- **Simmons Citrate Agar** (Manufacturer: HIMEDIA, Catalog no. M099S-100G) was used to identify the Gram-negative bacteria that are able to utilize citrate as the sole source of carbon.
- **Urea Agar Base** (Manufacturer: BD, Catalog no. 211795**)** was used to identify the Gram-negative bacteria that are able to produce Urease.
- **Triple Sugar Iron (TSI) Agar** (Manufacturer: BD, Catalog no. 226540**)** was used to identify the Gram-negative bacteria that are able to ferment sugars and produce hydrogen sulfide gas.
- **MR-VP Medium** (Manufacturer: BD, Catalog no. 216300) used for the differentiation of bacteria (especially Enterobacteriaceae) by means of the methyl red and Voges-Proskauer tests.

- **Brain Heart Infusion Broth** (Manufacturer: BD, Catalog no. 299070) was used for cultivating bacterial isolates and for preservation.
- **Peptone Type 1** (Manufacturer: HIMEDIA, Catalog no. RMG667-500G) was used for cultivation of the bacterial isolates and indole test.
- **Gram Stains-Kit** (Manufacturer: HIMEDIA, Catalog no. K001L-1KT) was used for differentiating between Gram positive and Gram-negative bacteria.

2.1.3. Miscellaneous reagents

- **Kovac's Indole Reagent** (Manufacturer: HIMEDIA, Catalog no. R008-100ML) was used for bacterial characterisation using the Indole test.
- **Phosphate Buffer Saline (PBS)** (Manufacturer: HIMEDIA, Catalog no. R008-100ML) was used to make bacterial smear for Gram staining.
- **Nuclease Free Water (NFW) (**Manufacturer: GeNei, Catalog no. 61215110001A**)** was used in DNA extraction and in the preparation of master mix for PCR reactions.
- **Saline Water (**Manufacturer: Biomerieux, Catalog no. 1204**)** was used in the inoculation preparation for the identification of bacterial isolates via the VITEK II automated system.
- **Glycerol (**Manufacturer: Fisher Scientific, Catalog no. G33-500**)** was used for the preservation of the bacterial isolates.
- **Voges Proskauer-A Reagent Droppers (α- Naphthol 5% in alcohol) (**Manufacturer: BD, Catalog no. 261192**)** was used in the biochemical characterisation of bacteria by VP test.
- **Voges Proskauer-B Reagent Droppers (KOH 40% in distilled water) (**Manufacturer: BD, Catalog no. 261193**)** was used in the biochemical characterisation of bacteria by VP test.
- **Methyl Red Indicator (**Manufacturer: HIMEDIA, Catalog no. I007-125ML**)** was used in the biochemical characterisation of bacteria by MR test.

2.1.4. Scientific Instruments Used

- **Nanodrop ND-1000 spectrophotometer** (Manufacturer: Nanodrop technologies, Inc)
- **ESCO Class II Biosafety cabinet** (Manufacturer: ESCO)

- **VITEK II Compact automated system** (Manufacturer: Biomerieux, Catalog no. 17868)
- **DensiCHEK plus** (Manufacturer: Biomerieux)
- **Olympus Microscope** (Manufacturer: Olympus, Catalog no. CH20ci)
- **MMM Friocell BOD incubator** (Manufacturer: Friocell, Catalog no. E14195)
- **Mettler Toledo ME104 weighing balance** (Manufacturer: Mettler Toledo, Catalog no. 2739261042)
- **ESCO Lexicon II ULT Freezer** (Manufacturer: ESCO, Catalog no. UUS-597A-1- 5D-SS)
- **Meta-lab scientific laboratory autoclave** (Manufacturer: Meta-lab Scientific, Catalog no. MSI-75)
- **Eppendorf centrifuge 5424** (Manufacturer: Eppendorf, Catalog no. 000842)
- **Eppendorf Nexus Gradient Thermal Cycler** (Manufacturer: Eppendorf)
- **Abdos LED Digital dry bath** (Manufacturer: ABDOS, Catalog no. E11330)
- **BR BIOCHEM Life sciences UV Ultra Dual with Viz Transilluminator** (Manufacturer: BR BIOCHEM Life sciences, Catalog no. UV6502-VIZ)
- **LABCONCO™ CentriVap DNA concentrator** (Manufacturer: LABCONCO)

2.1.5. Laboratory ware for Microbial Culture

- **90 mm Disposable Petri plates** (Manufacturer: Himedia, Catalog no. PW1132)
- **Borosil Test tubes** (Manufacturer: Borosil)
- **Borosil measuring cylinder- 1000 ml and 100 ml** (Manufacturer: Borosil)
- **Borosil Reagent bottles- 500ml and 250 ml** (Manufacturer: Borosil)
- **Inoculation Needle** (Manufacturer:Tarsons, Catalog no. 920071)
- **1µl Disposable Inoculation loops** (Manufacturer: Tarsons, Catalog no. 920061)
- **10µl Disposable Inoculation loops** (Manufacturer: Tarsons, Catalog no. 920061)

2.1.6. Miscellaneous Materials

- **Cavity Slide (**Manufacturer: Himedia , Catalog no. GW090-1PK**)**
- **Micro centrifuge tube 1.5 ml** (Manufacturer: Tarsons, Catalog no. 500016)
- **PCR tube 0.2ml** (Manufacturer: BioRad, Catalog no. TLS0801)

- **Tough tags** (Manufacturer: Microtube, Catalog no.T-SPOTS-B)
- **Bemis Parafilm 'M' Laboratory film** (Manufacturer: Bemis, Catalog no. PM996)

2.1.7. Polymerase Chain Reaction reagents

- **Forward and Reverse primers** (Manufacturer: Eurofins genomics)
- **Accuprime™ PCR Buffer I** (Manufacturer: Invitrogen, Catalogno.55188)
- **Accuprime™ PCR Buffer II** (Manufacturer: Invitrogen, Catalogno.55189)
- **Accuprime™ Taq DNA polymerase, High fidelity** (Manufacturer: Accuprime, Catalogno. 12346-086)
- **Promega PCR Master mix, 2X** (Manufacturer: PROMEGA, Catalog no. M750B)
- **Nuclease Free Water (NFW)** (Manufacturer: GeNei, Catalog no. 61215110001A)
- **Hi-Di Formamide** (Manufacturer: Applied Biosystems, Catalog no. 15803570)

2.1.8. Agarose Gel electrophoresis reagents

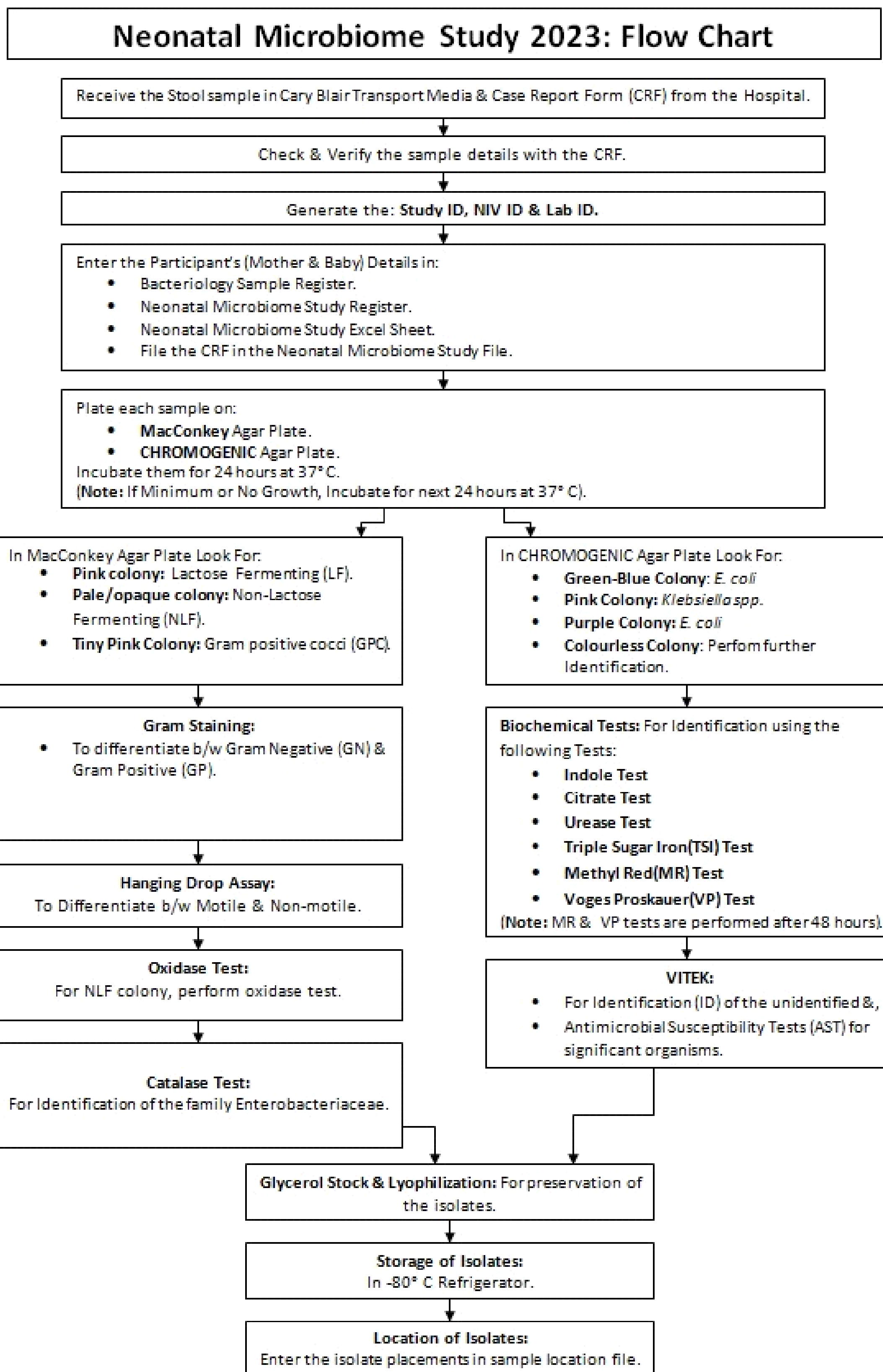
- **SYBR Gold nucleic acid gel stain** (Manufacturer: Invitrogen by Thermo Fisher Scientific, Catalog no. S11494)
- **100bp DNA ladder** (Manufacturer: Invitrogen by Thermo Fisher Scientific, Catalog no. 15628019)
- **6X DNA Loading dye** (Manufacturer: Thermo Scientific, Catalog no. R0611)
- **50X TAE Electrophoresis Buffer** (Manufacturer: thermo scientific, Catalog no. B49)
- **Agarose** (Manufacturer: MP, Catalog no. 180720)

2.1.9. PCR Product purification and sequencing reagents

- **QIAGEN® QIAquick PCR purification kit** (Manufacturer: QIAGEN, Catalog no. 28106)
- **Big Dye® Terminator v1.1,v3.1 5X Sequencing Buffer** (Manufacturer: Applied Biosystems, Catalog no. 4336697)
- **DyeEx® 2.0 Spin Kit (250)** (Manufacturer: QIAGEN, Catalog no. 63206)

2.2. Methodologies Used in the Study

The following flow chart was made to process each fecal specimen.



2.2.1. Sample Verification and Documentation

- Mother-baby pairs of C- section and vaginal delivery were recruited in collaboration with the clinical partner.
- Demographic information, mode of delivery and use of prophylactic antibiotics were recorded from the case report form (CRF).
- Fecal specimens were received in Cary Blair Transport Media from the clinical partner.
- After receiving, verification of the specimen details was done with the CRF.
- Study ID, NIV ID & Lab ID were generated for records.
- Entry of participant's (Mother & Baby) details were done in Bacteriology Sample Register, Neonatal Microbiome Study Register and, Neonatal Microbiome Study Excel Sheet.
- CRFs were filed in the Neonatal Microbiome Study File.

2.2.2. Sample Processing

After verification and documentation, samples were processed in the following manner.

- Each sample was plated on MacConkey Agar and CHROMOGENIC Agar plate, and were incubated at 37° C for 24 hours. When there was minimum or no growth, it was incubated for the next 24 hours.

2.2.3. Isolation of the bacterial colonies

Following incubation, plates were screened for isolation of the colonies based on their colony characteristics mentioned below in *Table 2.1* and *Table 2.2.*

Table 2.1 Characterisation of Lactose fermenting and non-lactose fermenting on MacConkey Agar Plate.

MacConkey Agar Plate
• Pink colony: Lactose Fermenting (LF).
• Pale/opaque colony: Non-Lactose Fermenting (NLF).
• Tiny Magenta Pink Colony: Gram positive cocci (GPC).

Table 2.2 Characterisation of bacteria based on color production on Chromogenic Agar Plate.

CHROMOGENIC Agar Plate
• Green-Blue Colony: *E. coli*
• Pink Colony: *Klebsiella pneumoniae.*
• Purple Colony: *E. coli*
• Colourless Colony: Perform further Identification.

2.2.4. Characterisation and Identification of the bacterial colony

After screening the plates, characterisation and identification of the bacterial colonies were done by following methods.

2.2.4.1. Gram Staining

Gram staining was performed in order to differentiate between Gram positive and Gram-negative bacteria.

2.2.4.1.1. Smear preparation

- A drop / loopful of PBS was placed onto a clean slide.
- A colony with a sterile loop was picked and mixed with PBS on the slide in a circular motion to make a thin, uniform bacterial smear.
- The smear was air dried and heat fixed using a spirit lamp.
- The slide was left to cool down.

2.2.4.1.2. Staining Procedure

- Primary stain: A few drops of Crystal Violet was added on the smear and kept for 1 minute, rinsed with water.
- Mordant: A few drops of Gram's Iodine were added and kept for 1 minute, rinsed with water.
- Decolorizer: Alcohol wash was slowly given for 10 – 30 seconds and was immediately rinsed with water.
- Counterstain: Finally, safranin was added to the smear and kept for 1 minute, rinsed with water.
- The slide was left to dry for a few minutes.

- After drying a drop of cedar wood oil was added to the dried smear.
- The slide was placed under the oil immersion objective of the microscope and the cells were observed.

2.2.4.2. Hanging Drop Motility Assay

Hanging drop motility assay was performed for the differentiation of motile and non-motile bacteria.

2.2.4.2.1. Preparation of bacterial suspension

- 200 µl PBS was added into the Eppendorf tube.
- From a pure culture plate, 3-5 colonies were picked, added and mixed to the PBS with a disposable loop to make a suspension.
- The suspension was gently vortexed to get a uniform suspension

2.2.4.2.2. Slide Preparation

- A loopful of bacterial suspension was placed in the center of a clean cover slip.
- Using a piece of parafilm, a stick ring was made and placed around the depression of the cavity slide.
- The cavity slide with the stick ring was placed over the coverslip in such a way that the cover slip got stuck to the ring on the slide.
- Immediately the slide was turned over so that the drop of bacterial suspension hung on the lower surface of the cover slip.

2.2.4.2.3. Microscopic Observation

- The drop was initially focused on the edge of the drop under the low power objective (10X) of the compound microscope.
- Once the edge was located, the cells were observed under the high-power objective (40 X).

2.2.4.3. Biochemical Tests

For the identification of a bacterial colony, the following biochemical tests mentioned in the *Table 2.3* were performed.

Table 2.3 Biochemical Tests and their protocol.

Name of the test	Procedure
Indole Test	• A sterilized test tube containing 3 ml of peptone water was taken. • The tube was inoculated by taking a well isolated colony from 18 to 24 hrs culture. • Incubated the tube at 37°C overnight. • 0.5 ml of Kovac’s reagent was added to the broth culture. • Observed the presence or absence of a ring. • Positive Indole test was indicated by the formation of pink to cherry red ring and no colour change was observed in case of negative test.
Citrate Test	• The surface of a citrate agar slant was streaked back and forth with a light inoculum picked a well-isolated colony. • The tube was incubated aerobically at 37°C overnight. • Positive citrate test was indicated by the color change from green to blue along the slant and no colour change was observed in case of negative test.
Urease Test	• The surface of a urea agar slant was streaked back and forth with a light inoculum picked from the center of a well-isolated colony. • The tube was incubated aerobically at 37°C for 24 hours. • Positive urease test was indicated by the color change from light yellow to bright pink color along the slant and no colour change was observed in case of negative test.

Triple Sugar Iron (TSI) Test	• With a straight inoculation needle, the top of a well-isolated colony was touched. • TSI media was inoculated by first stabbing through the center of the medium just short of the bottom of the tube and then the surface of the agar slant was streaked. • The tube was incubated at 37°C overnight. • **An alkaline/acid (red slant/yellow butt) reaction:** Indicated glucose fermentation only. • **An acid/acid (yellow slant/yellow butt) reaction:** Indicated glucose, lactose and/or sucrose fermentation. • **An alkaline/alkaline (red slant, red butt) reaction:** Indicated the absence of carbohydrate fermentation results. • **Gas production**: Bubbles or cracks in the agar indicated the production of gas (formation of CO2) • **Blackening of the medium: indicated production of** H2S.
Methyl Red (MR) Test	• A sterilized test tube containing 3 ml of MR broth was taken. • The tube was inoculated by taking a well isolated colony from the cultured plate. • The tube was incubated aerobically at 37°C for 48 hours. • After 48 hours of incubation, 2 to 3 drops of methyl red indicator were added to the tube. • Positive MR test was indicated by the formation of red colour and formation of yellow colour was observed in case of negative test.
Voges Proskauer (VP) Test	• A sterilized test tube containing 3 ml of VP broth was taken. • The tube was inoculated by taking a well isolated colony from the cultured plate.

	• The tube was incubated aerobically at 37°C for 48 hours. • 2-3 drops of VP-A (5% alpha-naphthol) reagent were added, and mixed well to aerate. • 2-3 drops of VP-B (40% potassium hydroxide) reagent were added, and mixed well to aerate. • The tube vigorously shaken during the 30-min period. • Positive VP test was indicated by the formation of a pink-red color at the surface within 30 min and no colour change was observed in case of negative test.

2.2.4.4. VITEK II Compact Automated Identification and Anitimicrobial Susceptibility Testing (AST)

Further identification and AST of some of the bacterial isolates were done by VITEK II compact automated system as per the following protocol.

- The room temperature was set to 22°C, the VITEK machine and desktop were turned on with user credentials.
- VITEK cards were retrieved from the refrigerated storage.
- The fresh cultured plates were taken out from the incubator.
- Gram negative (GN) bacterial identification was performed using a GN ID card.
- For AST of lactose-fermenting (LF) bacteria, Card No. AST405 was used, and for the AST of non-lactose-fermenting (NLF), card No. AST406 was used.
- Three plastic tubes were taken, first labelled as control, second with ID, and the third AST.
- 3 mL saline water was added to all the three tubes.
- The first tube i.e. the control tube's optical density (OD) was adjusted to 0.00McFarland standard.
- Isolated colonies were picked from quadrant 4 of the fresh cultured plate and added to the second test tube to make a bacterial suspension.
- The turbidity of the suspension was adjusted between 0.50–0.63 McFarland standard required for the GN bacteria.
- From the above 3 ml bacterial suspension, 145 µl was transferred to the AST test tube required to make a suspension for AST test.

- The ID card was inserted into the second tube while the AST card was inserted to the third tube.
- Prepared cassettes were inserted into the VITEK machine's filler section and the machine was operated using the VITEK software.

This process ensured accurate identification and antibiotic sensitivity determination of bacterial isolates.

2.2.5. Genotypic assays for determining antibiotic resistant genes in the bacterial isolates

2.2.5.1. DNA extraction via Thermal lysis method

DNA extraction of the bacterial isolates was done by a physical method i.e. thermal lysis as per the following protocol.

- Before starting the procedure, the Dry bath was set to a temperature of 100°C.
- Two microcentrifuge tubes were labelled. One with the specimen ID another with EC (Extraction Control for which only NFW was used).
- 300 µl of NFW was added into both tubes.
- A generous amount of bacterial colonies was added from the NA plate using a disposable inoculating loop to the respective tubes to get a turbid bacterial suspension.
- The suspensions were mixed well by vortexing.
- The tubes were kept in the dry bath heated at 100° C for 10 min.
- The tubes were spinned to remove any droplet inside the lid.
- The tubes were then centrifuged at 14000 rpm for 3 min.
- The supernatant was carefully transferred into a fresh microcentrifuge tube without disturbing the pellet (The pellet was discarded).
- Finally, the supernatant containing the DNA was stored at -20°C, and was used as the template for further PCR procedures.

2.2.5.2. Nanodrop spectrophotometer for quantification of the DNA

- The concentration of the extracted DNA was quantified using the Nanodrop ND-1000 Spectrophotometer.
- NFW was used as a blank.

- 2µl of the extracted DNA was loaded on the nanodrop instrument and the readings were recorded for every extracted DNA.

2.2.5.3. End Point PCR

2.2.5.3.1. Oligonucleotide primers reconstitution

To get ready for the polymerase chain reaction (PCR), a stock solution containing 100 pmol/µl of primers and probe was made first. A working stock solution with a concentration of 10 pmol/µl was then made using this stock solution. After adding NFW to the primer vials, the mixture was thoroughly vortexed to guarantee total dissolution and homogeneity. To reduce freeze-thaw cycles, the reconstituted primers were separated into small aliquots. After that, the aliquots were kept in storage at the suggested -20°C temperature to preserve their stability and guard against deterioration. Each aliquot was properly labelled to facilitate simple identification. *Table 2.4* lists the Oligonucleotide name, sequence and their expected amplicon size used in the End Point PCR.

Table 2.4 Oligonucleotide name, sequence, and their expected amplicon size.

Sr. No.	Oligo Name	Sequence	Expected amplicon size (bp)	Reference
1.	*TEM-1 & TEM-2*	F-CATTTCCGTGTCGCCCTTATTC	800	(Dallenne et al., 2010)
		R-CGTTCATCCATAGTTGCCTGAC		
2.	*SHV*	F- AGCCGCTTGAGCAAATTAAAC	713	
		R- ATCCCGCAGATAAATCACCAC		
3.	*OXA-1*	F-GGCACCAGATTCAACTTTCAAG	564	
		R-GACCCCAAGTTTCCTGTAAGTG		
4.	*KPC*	F-CATTCAAGGGCTTTCTTGCTGC	538	
		R- ACGACGGCATAGTCATTTGC		
5.	*CTX-M group 1*	F-TTAGGAARTGTGCCGCTGYA	688	
		R-CGATATCGTTGGTGGTRCCATb		
6.	*NDM-1*	F-ACCGCCTGGACCGATGACCA	264	(Zarfel et al., 2011)
		R-GCCAAAGTTGGGCGCGGTTG		

2.2.5.3.2. End Point PCR preparation

End point PCR for all the following targets with their cycling conditions were performed as per their given protocol. The reaction mixture was prepared. Depending on how many reactions were to be performed, the following mix components were determined.

Table 2.5 Concentration of Various Reagents Used in PCR using Accuprime Taq DNA Polymerase.

Sr. No.	Reagents	Concentration
1.	Accuprime™ PCR Buffer I/II	10X
2.	Forward Primer	10 pmol/µl
3.	Reverse Primer	10 pmol/µl
4.	AccuPrime Taq DNA Polymerase, High Fidelity	2.5U/µl

Table 2.6 Concentration of Various Reagents Used in PCR using Promega Master Mix Kit.

Sr. No.	Reagents	Concentration
1.	Promega Master Mix	2X
2.	Forward Primer	10 pmol/µl
3.	Reverse Primer	10 pmol/µl

Table 2.7.1 End Point PCR Protocol for gyrA

Target- *gyrA*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	10X Buffer (II)	2.5
2.	Forward Primer 10 pmol.	2.5
3.	Reverse Primer 10 pmol.	2.5
4.	Enzyme	0.5
5.	NFW	13
6.	DNA	4
7.	Total	25

Table 2.7.2 End Point PCR cycling condition for gyrA

PCR Cycling condition for ***gyrA***			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	94	10 mins	30
Denaturation	94	30 secs	
Annealing	66	60 secs	
Elongation	72	90 secs	
Final Elongation	72	7 mins	

Table 2.8.1 End Point PCR Protocol for TEM1 and TEM 2

Target- ***TEM 1 and TEM 2***		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	10X Buffer (II)	2.5
2.	Forward Primer 10 pmol.	1
3.	Reverse Primer 10 pmol.	1
4.	Enzyme	0.5
5.	NFW	17.5
6.	Hi-Di Formamide	0.5
6.	DNA	2
7.	Total	25

Table 2.8.2 End Point PCR cycling condition for TEM1 and TEM 2

PCR Cycling condition for ***TEM 1 and TEM 2***			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	95	15 mins	35
Denaturation	94	30 secs	
Annealing	59	90 secs	
Elongation	72	90 secs	
Final Elongation	72	7 mins	

Table 2.9.1 End Point PCR Protocol for SHV

Target- *SHV*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	10X Buffer (II)	2.5
2.	Forward Primer 10 pmol.	1
3.	Reverse Primer 10 pmol.	1
4.	Enzyme	0.5
5.	NFW	17.5
6.	Hi-Di Formamide	0.5
6.	DNA	2
7.	Total	25

Table 2.9.2 End Point PCR cycling condition for SHV

PCR Cycling condition for *SHV*			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	95	15 mins	
Denaturation	94	30 secs	
Annealing	59	90 secs	35
Elongation	72	90 secs	
Final Elongation	72	7 mins	

Table 2.10.1 End Point PCR Protocol for NDM-1

Target- *NDM-1*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	10X Buffer (I)	2.5
2.	Forward Primer 10 pmol.	1
3.	Reverse Primer 10 pmol.	1
4.	Enzyme	0.5
5.	NFW	17
6.	Hi-Di Formamide	1
6.	DNA	2
7.	Total	25

Table 2.10.2 End Point PCR cycling condition for NDM-1

PCR Cycling condition for *NDM-1*			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	94	5 mins	35
Denaturation	95	30 secs	
Annealing	58	30 secs	
Elongation	72	30 secs	
Final Elongation	72	10 mins	

Table 2.11.1 End Point PCR Protocol for KPC

Target- *KPC*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	Promega PCR Master mix, 2X	12.5
2.	Forward Primer 0.2 pmol.	2.5
3.	Reverse Primer 0.2 pmol.	2.5
4.	NFW	3.5
5.	DNA	4
6.	Total	25

Table 2.11.2 End Point PCR cycling condition for KPC

PCR Cycling condition for *KPC*			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	94	10 mins	35
Denaturation	94	40 secs	
Annealing	60	40 secs	
Elongation	72	1 min	
Final Elongation	72	7 mins	

Table 2.12.1 End Point PCR Protocol for OXA-1

Target- *OXA-1*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	Promega PCR Master mix, 2X	12.5
2.	Forward Primer 10 pmol.	1
3.	Reverse Primer 10 pmol.	1
4.	NFW	6.5
5.	DNA	4
6.	Total	25

Table 2.12.2 End Point PCR cycling condition for OXA-1

PCR Cycling condition for *OXA-1*			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	95	15 mins	35
Denaturation	94	30 secs	
Annealing	55	90 secs	
Elongation	72	90 secs	
Final Elongation	72	7 mins	

Table 2.13.1 End Point PCR Protocol for CTX-M1

Target- *CTX-M1*		
Sr. No.	**Reagents**	**Volume for single reaction(µl)**
1.	Promega PCR Master mix, 2X	12.5
2.	Forward Primer 10 pmol.	1
3.	Reverse Primer 10 pmol.	1
4.	NFW	7
5.	DNA	3
6.	Hi-Di Formamide	0.5
7.	Total	25

Table 2.13.2 End Point PCR cycling condition for CTX-M1

PCR Cycling condition for *CTX-M1*			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	94	10 mins	35
Denaturation	94	40 secs	
Annealing	60	40 secs	
Elongation	72	1 min	
Final Elongation	72	7 mins	

After making the PCR Master mix and aliquoting it into 500μl PCR tubes, the template DNA was added. There was also a no-template control (NTC). To avoid air bubbles, the tubes were centrifuged after that. The Eppendorf Nexus Gradient Thermal Cycler was filled with the tubes. The software was configured with the cycle conditions. After the tubes were inserted into the apparatus, the PCR reaction was carried out.

2.2.5.3.3. Agarose gel electrophoresis

DNA fragments were separated and visualised using agarose gel electrophoresis. By dissolving agarose powder in 1X TAE (Tris-Acetate-EDTA) buffer, heating, and cooling to roughly 60°C, a 1.8% agarose gel was created. The mixture was mixed with 6 μl of SYBR Gold nucleic acid stain. Each amplified PCR product was loaded into the gel along with a 100 kb DNA ladder using 1 μl of the 6X loading dye. The gel was run at 70 V for 90 minutes, and the Genetix Geneview 645SC Gel documentation system was used to visualise and document the results.

2.2.6.2. PCR PURIFICATION AND SEQUENCING

2.2.6.2.1. QIAquick PCR product Purification from agarose gel

The QIAquick purification kit provides a quick and simple bind-wash-elute process for DNA purification of fragments up to 10 kb, with elution volumes ranging from 30 to 50 μl. The QIAquick spin column, which includes a silica membrane, is designed to purify DNA from both aqueous solutions and agarose gels.

Protocol: The DNA fragment was excised from the agarose gel using a clean, sharp scalpel. The gel slice was weighed in a colorless tube, and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg gel ~100 µl). The tube was incubated at 50°C for 10 minutes until the gel slice had completely dissolved. Next, one gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in a provided 2 ml collection tube or a vacuum manifold. The sample was applied to the QIAquick column and centrifuged at 13000 rpm for 1 minute, the flow-through was discarded, and the QIAquick column was returned to the same tube. 500 µl of Buffer QG was added to the QIAquick column and centrifuged again for 1 minute at 13000 rpm. The flow-through was discarded, and the QIAquick column was returned to the same tube.

Afterwards, 750 µl of Buffer PE was added to the column and incubated at room temperature for 5 minutes and then it was centrifuged at 13000 rpm for 1 minute in order to wash the column. The flow-through was discarded, and the column was returned to the same tube.

The QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane, and the column was centrifuged for 1 minute. The purified DNA was quantified using the Nanodrop ND-1000 Spectrophotometer and then stored at -20°C for further use.

2.2.6.2.2. Big Dye Terminator PCR

After the above purification, Big dye terminator PCR was performed using the following protocol and cycling conditions mentioned in *Table 2.13.1* and *Table 2.13.2.*

Table 2.14.1 Components of Sequencing reaction mixture for Big Dye Terminator.

Reagent	Forward	Reverse
Big Dye Terminator	4	4
Primer(F)	1	0
Primer (R)	0	1
NFW	1	1
DNA	4	4
Total	10	10

Table 2.14.2 PCR cycling conditions for the target CTX-M1.

PCR cycling conditions			
Steps	**Temp. in °C**	**Time**	**Cycles**
Initial Denaturation	96	1 min	25
Denaturation	96	10 secs	
Annealing	60	30 secs	
Elongation	60	4 min	

2.2.6.2.2. Dye-EX Purification

To perform the DNA purification, the following steps were performed:

- The spin column was gently vortexed to re-suspend the resin.
- Loosened the cap of the column a quarter turn (to avoid any vacuum inside the spin column).
- The bottom closure of the spin column was snapped off and placed in a 2 ml collection tube.
- Centrifuged for 3 minutes at 3500 rpm.
- The spin column was carefully transferred to a clean microcentrifuge tube.
- Slowly applied the sequencing reaction (10-20 µl) to the gel bed.
- Centrifuged for 3 minutes at 13000 rpm.
- Removed the spin column from the microcentrifuge tube.

2.2.6.2.3. Sanger Sequencing

Sanger sequencing replicates a DNA template in a reaction mixture that includes DNA polymerase, normal deoxynucleotides (dNTPs), and a small amount of fluorescently labelled chain-terminating dideoxynucleotides (ddNTPs). During DNA synthesis, the DNA polymerase integrates dNTPs and ddNTPs at random along the developing DNA chain. However, the presence of ddNTPs (2'-3'-dideoxynucleotide triphosphate) precludes continued DNA synthesis since it lacks the 3'-OH group required for continuing. The expanding strand reaches its end point. When subjected to a UV laser, each of the four different ddNTPs generates fluorescence at a specific wavelength, which can be detected and recorded by a sensor. The reaction, which starts at the primer sequence but ends at different bases, produces new DNA molecules of varied

lengths as a result. Analysing the terminated segments' sequence from shortest to longest will reveal the starting DNA sequence.

2.2.6.2.4. Sequence editing and BLAST analysis

Using chromatogram files in the Chromas software, the quality of the sequencing findings was assessed in order to spot possible sequencing errors and eliminate poor-quality areas. The DNASTAR’s Megalign and EditSeq tools were used to align and modify the sequences that were acquired by forward and reverse reactions. After sequence editing, the optimized sequence was sent to BLAST analysis for analysis. In order to confirm the quality of the edited sequence, Chromas, a chromatogram viewing software, was used to visualise the final sequence. Chromas offered a graphical depiction of the modified sequence matched to the matching chromatogram, enabling a thorough evaluation of the overall sequence integrity and base-calling quality. A software called MegAlign was used to import the raw sequencing data. The chromatograms underwent analysis and alignment with reference sequences in order to detect possible discrepancies, such errors in base-calling or unclear sections. MegAlign made it easier to see the resulting sequence and compare it with known sequences, which made it easier to spot and fix any errors. Sequence editing software EditSeq was used to further improve the altered sequence after it was first analysed in MegAlign. By precisely modifying the sequence using EditSeq, it was possible to eliminate any lingering ambiguities, modify base-calling, and reduce low-quality sections. By using an iterative method, the cloned insert was accurately reproduced in a high-quality edited sequence.

Chapter 3 Results

3.1. Clinical Specimen

Fifteen Mother-baby pairs each of C-section and vaginal delivery along with their baby's follow-up (Ten from C-section and Five from Vaginal delivery) fecal samples were screened for the isolation and identification of aerobic Gram-negative bacteria.

3.2. Isolation of the bacterial colonies

Aerobic Gram-negative bacteria (*E. coli* and *K. pneumoniae*) were identified and isolated from all the fecal specimen of fifteen mother-baby pairs (each from C-section and vaginal delivery) along with their baby's follow-up specimens (Ten from C-section and Five from Vaginal delivery) cultivated on MacConkey Agar and Chromogenic Agar as per protocol mentioned in **2.2.2.** and **2.2.3.**
The total number of isolates are mentioned in the *Table 3.1.* The morphology and the colony characteristics of *E. coli* and *K. pneumoniae* are depicted in *Fig. 3.1-3.4.*

Table 3.1 Total number of E. coli and *K. pneumoniae isolated from fifteen C-section and vaginal delivery mother-baby pairs including the baby's follow-up fecal specimens.*

Sr. No.	Mode of delivery	Organism	No. of isolates from mother's specimen	No. of isolates from baby's meconium	No. of isolates from baby's follow up specimen	Total number of isolates
1.	C-section	*E. coli*	16	0	10	26
		K. pneumoniae	9	0	10	19
2.	Vaginal delivery	*E. coli*	16	0	5	21
		K. pneumoniae	7	0	3	10

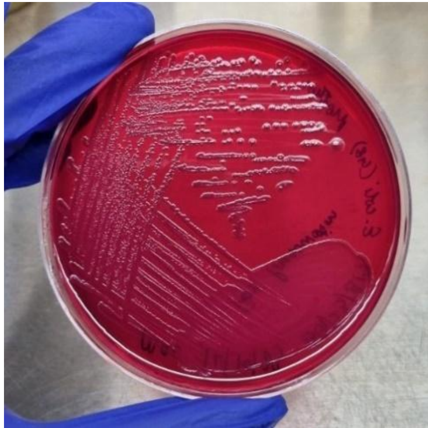


Fig. 3.1 (Mac Agar Plate)

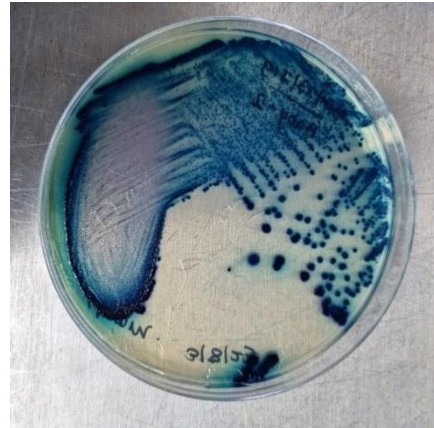


Fig. 3.2 (ChromAgar Plate)

Fig. 3.1 & 3.2 Morphology and colony characteristics of a representative E. coli cultivated from a fecal specimen on a Mac and Chrom agar plate.

Fig. 3.3 (Mac Agar Plate)

Fig. 3.4 (ChromAgar Plate)

Fig. 3.3 & 3.4 Morphology and colony characteristics of a representative K. pneumoniae cultivated from a fecal specimen on a Mac and Chrom agar plate.

3.3. Characterisation of bacterial colonies

All the isolates of *E. coli* and *K. pneumoniae* from the fecal specimen of fifteen mother-baby pairs (each from C-section and vaginal delivery) along with their baby's follow-up (Ten from C-section and Five from Vaginal delivery) were characterised and identified by the Biochemical tests along with few identified via VITEK II compact automated identification system as per the protocol mentioned in **2.2.4.**

The biochemical test results for characterizing a representative *E. coli* and *K. pneumoniae* are listed in *Table 3.2* and, the corresponding figures for each species are displayed in *Figs. 3.5* and *3.6.*

Table 3.2. Biochemical test results from a representative E. coli and K. pneumoniae.

Biochemical Tests									
Sr. No	**Type of Organism**	**Gram's Stain**	**Motility**	**Indole**	**Citrate**	**Urease**	**Triple Sugar Iron (TSI)**	**Methyl Red (MR)**	**Voges-Proskauer (VP)**
1.	*Escherichia coli*	Gram negative rods	Motile	Positive	Negative	Negative	Acid/Acid + Gas	Positive	Negative
2.	*Klebsiella pneumoniae*	Gram negative rods	Non-motile	Negative	Positive	Positive	Acid/Acid + Gas	Negative	Positive

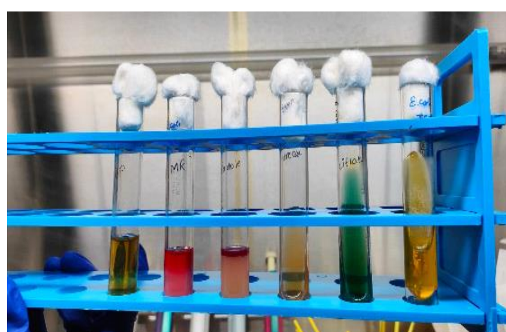


Fig. 3.5 Biochemical Identification of E. coli

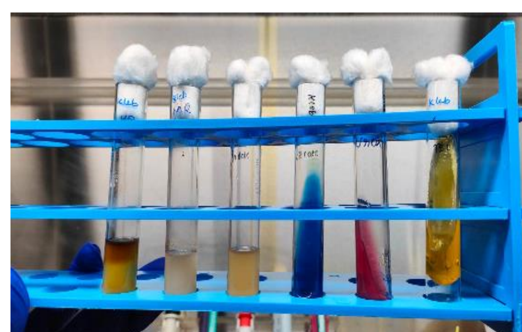


Fig. 3.6 Biochemical Identification of K. pneumoniae

Few organisms identified by VITEK II compact automated identification system are listed in the *Table 3.3*

Table 3.3 VITEK II Compact Automated Identification results from for the unidentified organism.

Sr. No.	**Type of organism Identified**	**Organism found in**	**Probability of Identification (in %)**
1.	*E. coli*	Baby's meconium	99
2.	*Sphingomonas paucimobilis*	Baby's meconium	98
3.	*Kocuria kristinae*	Baby's meconium	99
4.	*Acinetobacter lwoffii*	Baby's meconium	99

3.4. VITEK II Compact AST

VITEK II compact automated AST was performed for four representative isolates (two from mother and two from baby's follow-up) as per protocol mentioned in **2.2.4.4.** and their results are listed in the *Table 3.4*

Table 3.4. VITEK II Compact Automated AST results for four representative isolates.

	Beta Lactamase															
	Synthetic Penicillins		Cephalosporin					Carbapenem			Aminoglycosides		Fluro Quinolones	Glycylcycline	Polymyxin	Cotrimoxazole
Organism	Amoxycillin/Clavulanic Acid	Piperacillin/Tazobactam	Cefuroxime	Cefuroxime Axetil	Ceftriaxone	Cefoperazone	Cefepime	Ertapenem	Imipenem	Meropenem	Amikacin	Gentamicin	Ciprofloxacin	Tigecycline	Colistin	Trimethoprim/Sulfamethoxazole
E. coli (Mother)	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S
E. coli (Mother)	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S
K.pneumoniae (Baby)	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S	R
K.pneumoniae (Baby)	S	S	R	R	R	S	R	S	S	S	S	S	S	S	S	S

*R: Resistant, S: Susceptible, I: Intermediate

3.5. Genotypic assays for determining antibiotic resistant genes in the bacterial isolates

3.5.1. DNA extraction via Thermal lysis method

DNA of 47 *E. coli* and 29 *K. pneumoniae* isolated from fifteen mother-baby pairs (each from C-section and vaginal delivery) along with their baby's follow-up (Ten from C-section and Five from Vaginal delivery) were extracted using the protocol of thermal lysis method mentioned in **2.2.5.1.**

3.5.2. Nanodrop spectrophotometer for quantification of the DNA

The concentrations of DNA extracted from both the isolates i.e. *E. coli* and *K. pneumoniae* (n=76, including fifteen C-sections and fifteen vaginal deliveries pairs along with their baby's follow-up specimens) ranged from 108.52 ng/ µl to 223.59 ng/µl with the average DNA purity (A260/280 ratio) of 1.99. All the quantification were performed using the protocol of nanodrop spectrophotometer mentioned in **2.2.5.2.**

3.5.3. End Point PCR

End point PCR was performed for all the isolates of *E. coli* and *K. pneumoniae* from fifteen mother-baby pairs of both C-section and vaginal delivery including the baby's follow-up isolates as per the protocol mentioned in **2.2.5.3.**

3.5.3.1. End Point PCR findings for C-section Isolates

End point PCR was performed for all the fifteen C-section mother-baby pairs including their baby's follow-up isolates. *Table 3.5* lists the end point PCR results for all the C-section isolates.

Table 3.5. Total number of E. coli and *K. pneumonia isolates tested positive for different antibiotic resistant genes from fifteen C-section mother-baby pairs including their baby's follow-up isolates.*

Sr.No.	**Isolates from C-section**	***TEM 1 & 2***	***SHV***	***NDM-1***	***OXA-1***	***KPC***	***CTX-M1***
1.	Mother's *E. coli*	5/16	1/16	0/16	2/16	0/16	8/16
2.	Baby's *E. coli* (Follow-up)	0/10	0/10	0/10	0/10	0/10	1/10
3.	Mother's *K. pneumoniae*	2/9	1/9	1/9	0/9	0/9	3/9
4.	Baby's *K. pneumoniae* (Follow-up)	2/10	6/10	2/10	3/10	0/10	2/10

Inferences from C-section isolates on Antibiotic resistant genes (ARG)

- Among Mother's *E. coli* isolates (n=16), the most abundant Antibiotic resistant gene (ARG) was found to be *CTX-M1* (8 positives), followed by *TEM-1 & 2* (5 positive) and *OXA-1* (2 positive) with *SHV* (1 positive) being the least among all. None of the isolates had *NDM-1* and *KPC* resistant gene.
- Among Baby's follow-up *E. coli* isolates (n=10), only one isolate had *CTX-M1* (1 positive) resistant gene.
- Among Mother's *K. pneumoniae* isolates (n=9) the most abundant ARG was found to be *CTX-M1* (2 positives), followed by *TEM-1 & 2* (3 positives) with *SHV* (1 positive), and *NDM-1* (1 positive) being the least among all. None of the isolates had *OXA-1* and *KPC* resistant gene.
- Among Baby's follow-up *K. pneumoniae* isolates, the most abundant ARG was found to be *SHV* (6 positives), followed by *OXA-1* (3 positives), with *TEM-1 & 2* (2 positives), *NDM-1* (2 positives), and *CTX-M1* (2 positives) being the least among all. None of the isolates had *KPC* resistant gene.

3.5.3.2 End Point PCR findings for Vaginal Delivery Isolates

End point PCR was performed for all the fifteen vaginal delivery mother-baby pairs including their baby's follow-up isolates. *Table 3.6* lists the end point PCR results for all the vaginal delivery isolates.

Table 3.6. Total number of E. coli and *K. pneumonia isolates tested positive for different antibiotic resistant genes from fifteen vaginal delivery mother-baby pairs including their baby's follow-up isolates.*

Sr. No.	Isolates from vaginal delivery	*TEM 1 & 2*	*SHV*	*NDM-1*	*OXA-1*	*KPC*	*CTX-M1*
1.	Mother's *E. coli*	8/16	2/16	1/16	2/16	0/16	4/16
2.	Baby's *E. coli* (Follow-up)	0/5	0/5	0/5	0/5	0/5	1/5
3.	Mother's *K. pneumoniae*	0/7	6/7	0/7	0/7	0/7	0/7
4.	Baby's *K. pneumoniae* (Follow-up)	1/3	2/3	1/3	1/3	0/3	1/3

Inferences from Vaginal delivery isolates on Antibiotic resistant genes (ARG)

- Among Mother's *E. coli* isolates (n=16), the most abundant Antibiotic resistant gene (ARG) was found to be *TEM-1 & 2* (8 positives), followed by *CTX-M1* (4 positives), *OXA-1* (2 positives) with *SHV* (2 positives) being the least among all. None of the isolates had *KPC* resistant gene.
- Among Baby's follow-up *E. coli* isolates (n=5), only one isolate had *CTX-M1* (1 positive) resistant gene.
- Among Mother's *K. pneumoniae* isolates (n=7), the most abundant ARG was *SHV* (6 positives).
- Among Baby's follow-up *K. pneumoniae* isolates(n=3), the most abundant ARG was found to be *SHV* (2 positives) followed by *TEM-1 & 2* (1 positive), *NDM-1* (1 positive), *OXA-1* (1 positive), and *CTX-M1* (1 positive) all being the least. None of the isolates had *KPC* resistant gene.
- One Ciprofloxacin resistant *K. pneumoniae* isolate from the baby's follow-up tested positive for *gyrA*.

Table 3.7 The table provides a breakdown of the isolates of E. coli and K. pneumoniae obtained from mothers and babies, highlighting the presence or absence of antibiotic resistance genes and multidrug resistance.

Sr. No.	Type of isolate	Total number of isolates	Total number of isolates without any ARG	Total number of isolates with at least one ARG	Total number of isolates with multiple ARGs (>1)
1.	Mother's *E. coli*	32	15 (6: C-section 9: Vaginal delivery)	7 (6: C-section 1: Vaginal delivery)	10 (4: C-section 6: Vaginal delivery)
2.	Baby's *E. coli* (Follow-up)	15	13 (9: C-section 4: Vaginal delivery)	2 (1:C-section 1: Vaginal delivery)	0
3.	Mother's *K. pneumoniae*	16	4 (3: C-section, 1: Vaginal delivery)	11 (5: C-section 6: Vaginal delivery)	1 (C-section)
4.	Baby's *K. pneumoniae* (Follow-up)	13	4 (3: C-section 1: Vaginal delivery)	4 (3: C-section 1: Vaginal delivery)	5 (4: C-section 1: Vaginal delivery)

Inference from mother's isolates (*E. coli and K. pneumonia*)

From mother's *E. coli* isolates (n=32),

- The total number of isolates without any ARG were 15 (6 from C-section and 9 from vaginal delivery).
- The total number of isolates with at least one ARG were 7 (6 from C-section and 1 from vaginal delivery).
- The total number of isolates with multiple ARGs were 10 (4 from C-section and 6 from Vaginal delivery).

From mother's *K. pneumoniae* isolates (n=16),

- The total number of isolates without any ARG were 4 (3 from C-section and 1 from vaginal delivery).
- The total number of isolates with at least one ARG were 11 (5 from C-section and 6 from vaginal delivery).
- There was only one isolates(C-section) with multiple ARGs.

Inference from baby's follow-up isolates (*E. coli and K. pneumonia*)

From baby's follow-up *E. coli* isolates (n=15),

- The total number of isolates without any ARG were 13 (9 from C-section and 4 from Vaginal delivery).
- The total number of isolates with at least one ARG were 2 (1 from C-section and 1 from Vaginal delivery).
- There were no isolates with multiple ARGs.

From baby's follow-up *K. pneumoniae* isolates (n=13),

- The total number of isolates without any ARG were 4 (3 from C-section and 1 from Vaginal delivery).
- The total number of isolates with at least one ARG were 4 (3 from C-section and 1 from Vaginal delivery).
- The total number of isolates with multiple ARGs were 5 (4 from C-section and 1 from Vaginal delivery).

3.5.4. Agarose gel electrophoresis

End point PCR were performed and the products were run in agarose gel electrophoresis as per protocol described in **2.2.5.3.3.** The End point PCR results for all the targets (*TEM-1 and 2, SHV, OXA-1, NDM-1, KPC, CTX-M1*) were confirmed by the gel images mentioned in the gel electrophoresis images in this section.

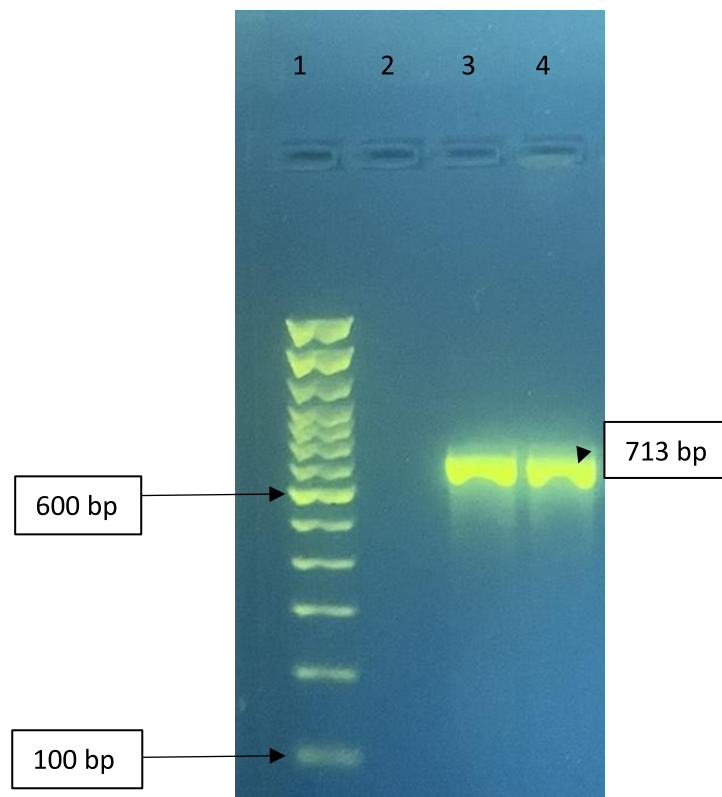


Fig. 3.7 SHV positive of a representative K. pneumoniae isolates from baby's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3 & 4: Isolates positive for SHV

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

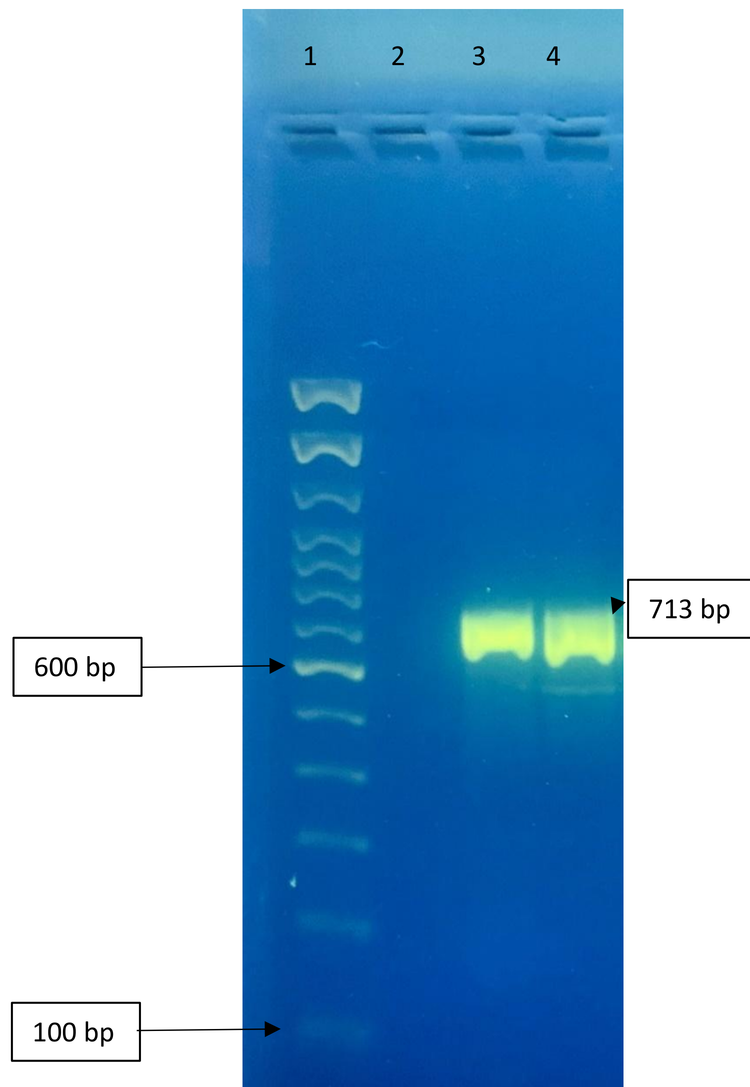


Fig. 3.8 SHV positive of a representative K. pneumoniae isolates from mother's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3 & 4: Isolates positive for SHV

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

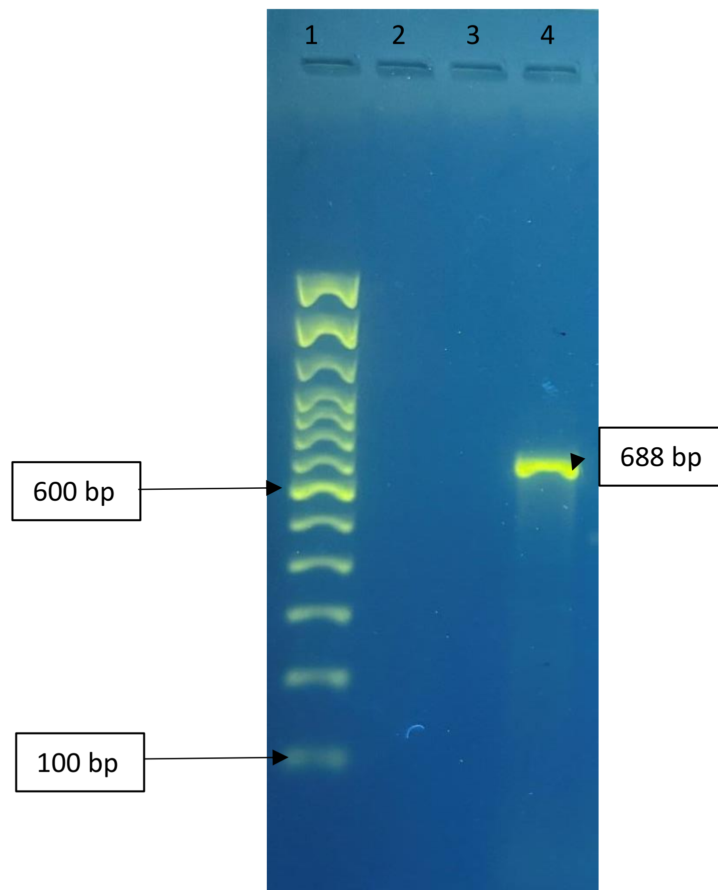


Fig. 3.9 CTX-M1 positive of a representative E. coli isolate from mother's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3: Isolate negative for CTX-M1

Lane 4: Isolates positive for CTX-M1

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

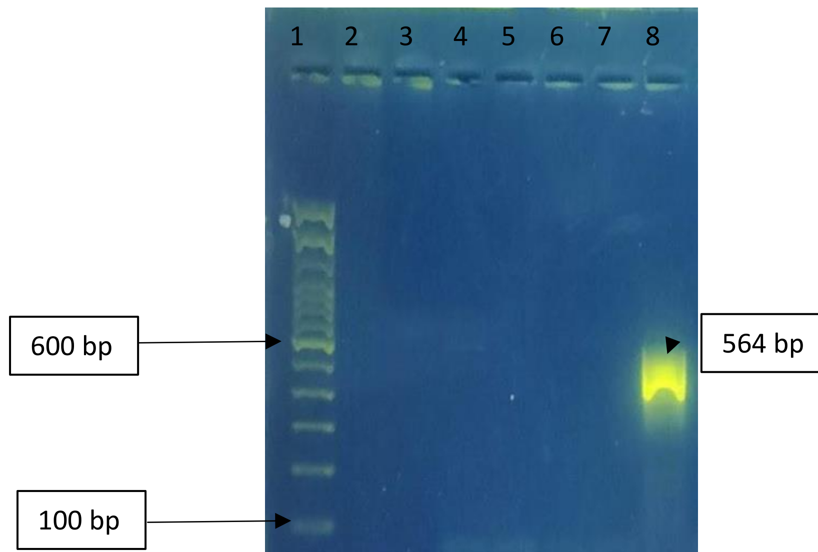


Fig. 3.10 OXA-1 positive of a representative E. coli isolate from mother's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3-7: Isolate negative for OXA-1

Lane 6: Isolates positive for OXA-1

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

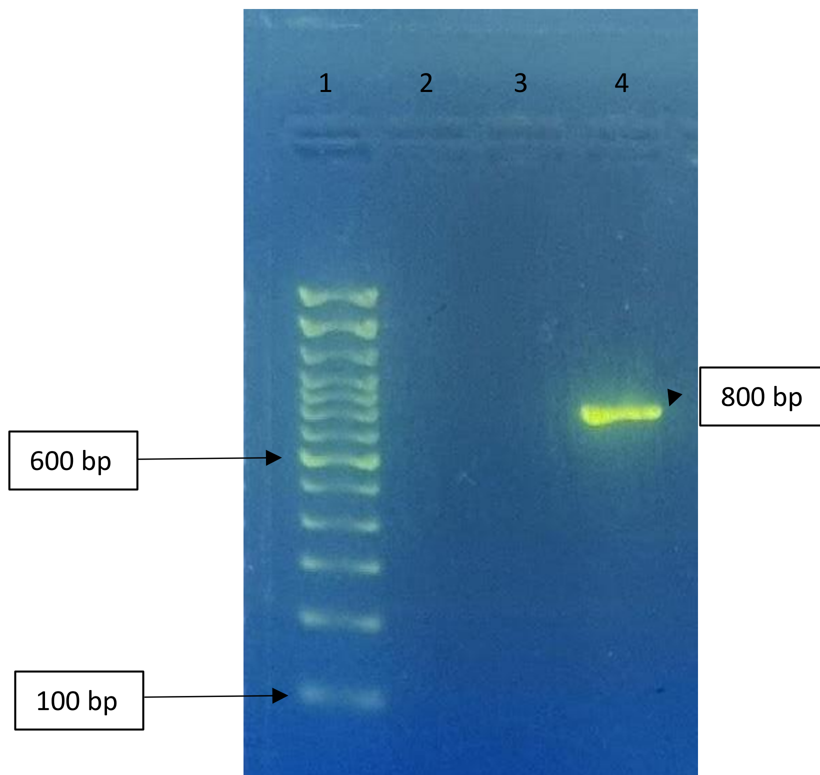


Fig. 3.11 TEM-1 & 2 positive of a representative E. coli isolate from mother's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3: Isolate negative for TEM-1 & 2

Lane 4: Isolate positive for TEM-1 & 2

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

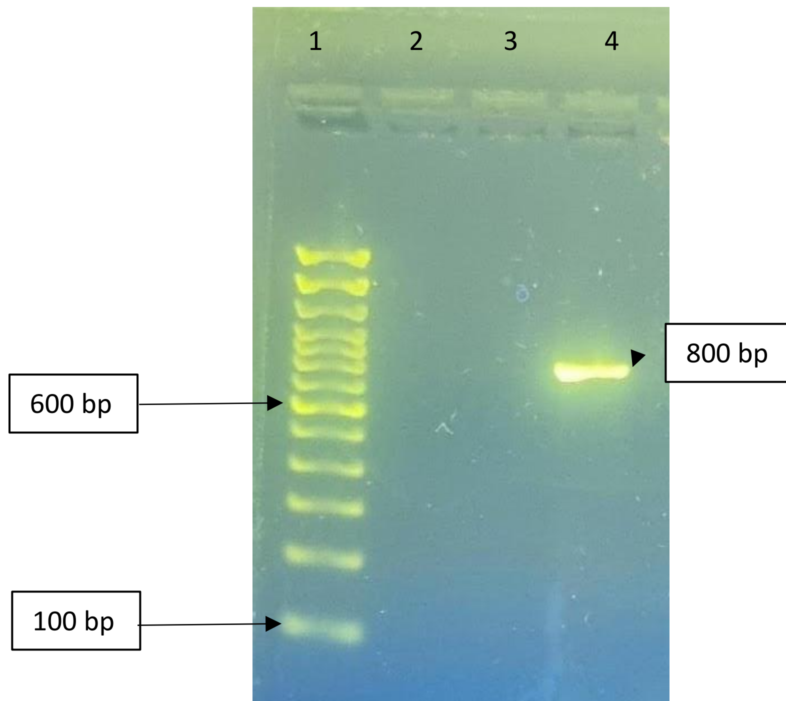


Fig. 3.12 TEM-1 & 2 positive of a representative K. pneumoniae isolate from baby's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3: Isolate negative for TEM-1 & 2

Lane 4: Isolate positive for TEM-1 & 2

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

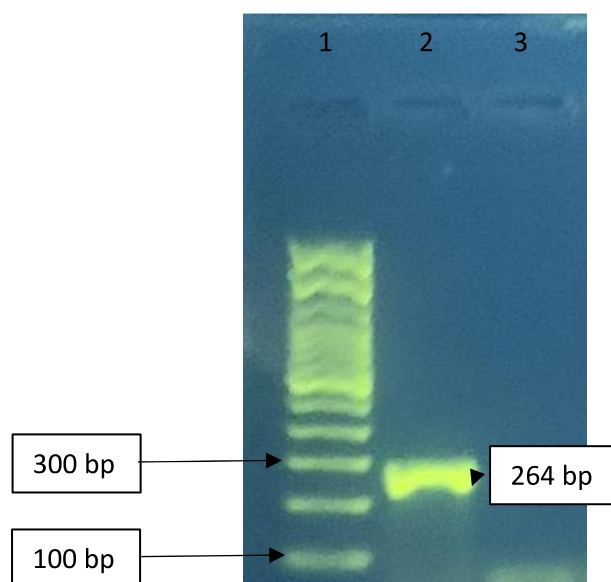


Fig. 3.13 NDM-1 positive of a representative K. pneumoniae isolate from baby's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: Isolates positive for NDM-1

Lane 3: NC

**Similar findings were noted for all the isolates (E. coli and K. pneumoniae) from mother and baby*

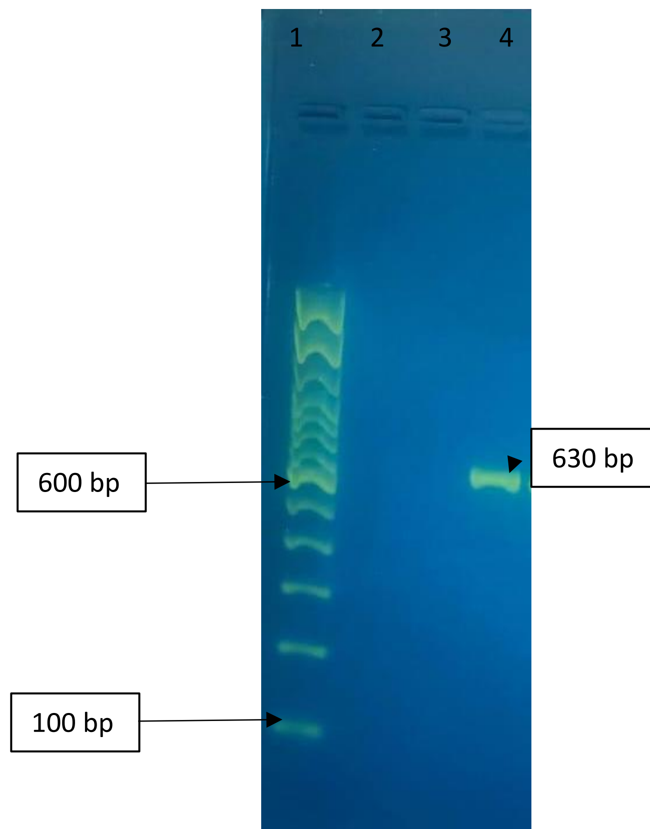


Fig. 3.14 gyrA positive of a representative K. pneumoniae isolate from baby's specimen.

Lane 1: 100bp DNA Ladder

Lane 2: NC

Lane 3: Isolate negative for gyrA

Lane 4: Isolate positive for gyrA

3.5.4. Sanger sequencing and BLAST analysis

The sequencing of the target *CTX-M1* was performed for two representative isolates as per protocol mentioned in **2.2.6.2.3**. To assure the reliability and accuracy, the sequenced data was edited as per section **2.2.6.2.4.**

The resulting sequence was compared with publicly available sequence databases to discover homologous or similar sequences.

For *E. coli:*

- The BLAST analysis of the sequencing findings for the target *CTX-M1* revealed 99.8%(Forward) identity with the sequence of *Escherichia coli* strain E74ECMO beta-lactamase CTX-M-15 isolate from the Enterobacteriaceae family
- The BLAST analysis of the sequencing findings for the target *CTX-M1* for *E. coli* revealed 99.7%(Reverse) identity with the sequence of *Shigella boydii* strain S 51 CTX-M family extended-spectrum class A beta-lactamase (*blaCTX-M*) gene isolate from the Enterobacteriaceae family

For *K. pneumoniae:*

- The BLAST analysis of the sequencing findings for the target *CTX-M1* of *E. coli* revealed 99.6%(Forward) identity with the sequence of *Shigella sonnei* strain S9 CTX-M family extended-spectrum class A beta-lactamase (*blaCTX-M*) gene isolate from the Enterobacteriaceae family.
- The BLAST analysis of the sequencing findings for the target *CTX-M1* of *E. coli* revealed 100%(Reverse) identity with the sequence of *Shigella boydii* strain S 51 CTX-M family extended-spectrum class A beta-lactamase (*blaCTX-M*) gene isolate from the Enterobacteriaceae family

Table 3.8 BLAST analysis of the sequencing result for the target CTX-M1(Forward and Reverse) for a representative of E. coli and K. pneumoniae.

Organism	**Target**	**% Identity**	**Max. Score**	**Total Score**	**Accession No.**
E. coli	CTX-M1(Forward)	99.8	1151	1151	KY640534.1
	CTX-M1(Reverse)	99.7	1144	1144	PP025719.1
K. pneumoniae	CTX-M1(Forward)	99.8	1146	1146	PP025716.1
	CTX-M1(Reverse)	100	1136	1136	PP025719.1

Chapter 4 Discussion

Discussion

The results shed light into the isolation and identification of aerobic gram-negative bacteria, comprising of *E. coli* and *K. pneumoniae*, from the clinical specimen of mother-baby pairs. The study included both C-section and vaginal delivery cases, with a focus on fecal specimens.

In the isolation of bacterial colonies, it was observed that *E. coli* and *K. pneumoniae* were present in all the fecal specimens of the mother-baby pairs, regardless of the mode of delivery. In C-section cases, *E. coli* was more common, with 16 isolates from the mother's specimen, while *K. pneumoniae* had 9 isolates. Similarly, in vaginal delivery cases, *E. coli* had 16 isolates compared to 7 isolates of *K. pneumoniae*. These findings suggest that *E. coli* is more abundant in both delivery modes.

The characterization of bacterial colonies involved biochemical tests and the use of the VITEK II compact automated identification system aided in identifying organisms that were initially unidentified like *Sphingomonas paucimobilis*, *Kocuria kristinae*, and *Acinetobacter Iwoffii*, along with their corresponding identification probabilities. This highlights the utility of the automated system in accurately identifying bacterial species.

The study also conducted VITEK II Compact Automated AST for four representative isolates. The results states the susceptibility or resistance of the isolates to different antibiotics. This information is crucial for understanding the antibiotic resistance patterns of *E. coli* and *K. pneumoniae* strains in the studied population.

Furthermore, genotypic assays were performed to determine antibiotic-resistant genes in the isolated bacteria. The DNA extraction method and quantification using a Nanodrop spectrophotometer ensured the availability of sufficient DNA for analysis. End point PCR was then conducted to detect specific genes associated with antibiotic resistance in *E. coli* and *K. pneumoniae* isolates. A total of 47 *E. coli* and 29 *K. pneumoniae* isolates were analyzed, including samples from C-section and vaginal delivery cases, as well as their corresponding baby's follow-up specimens. The identification of specific resistance genes in the isolates can provide valuable insights into the prevalence and potential spread of antibiotic resistance.

In the C-section group, the end point PCR analysis revealed the presence of various antibiotic-resistant genes in both *E. coli* and *K. pneumoniae* isolates. The commonly detected resistance genes in *E. coli* were *blaTEM*, *blaCTX-M*, and *blaSHV*, which are linked with resistance to beta-lactam antibiotics. These findings suggest a higher occurrence of antibiotic resistance in the C-section group. In contrast, the vaginal delivery group showed a different pattern of antibiotic-resistant genes. While the presence of *blaTEM*, *blaCTX-M*, and *blaSHV* genes was also observed in *E. coli* isolates, their frequency was lower compared to the C-section group. This finding indicates a potential difference in the antibiotic resistance profiles between the two delivery modes.

The observed differences in the pattern of antibiotic-resistant genes between the C-section and vaginal delivery groups could be attributed to various factors. One possible explanation is the difference in the maternal gut microbiota between the two groups. It is well understood that the mode of delivery can have an impact on the development of the infant's gut microbiota, which can therefore alter the transfer of antibiotic-resistant genes. To fully investigate the effects of this possible relationship, additional research is required.

The findings from the end point PCR analysis highlight the importance of monitoring antibiotic resistance in both C-section and vaginal delivery cases. The higher occurrence of antibiotic-resistant genes in the C-section group raises concerns about the potential transmission of resistance to newborns. This emphasizes the need for appropriate infection control measures and judicious use of antibiotics in the hospital setting. To clarify the underlying processes and determine the long-term consequences of these discoveries, further investigation is necessary.

Conclusion

In conclusion, the results this study shed light on the presence, distribution, and characteristics of aerobic Gram-negative bacteria, particularly *E. coli* and *K. pneumoniae*, in clinical specimens of mother-baby pairs. The study demonstrated that both C-section and vaginal delivery cases harbour these bacteria, with *E. coli* being more in number. However, *K. pneumoniae* showed a higher occurrence in C-section cases.

The biochemical tests and VITEK II compact automated identification system successfully identified the targeted bacterial species, while also revealing the presence of other organisms. This highlights the importance of employing advanced identification techniques for accurate bacterial characterization.

The antimicrobial susceptibility testing (AST) provided insights into the resistance profiles of the representative isolates. The genotypic assays further contributed to understanding the presence of antibiotic-resistant genes in the isolated *E. coli* and *K. pneumoniae* strains from the C-section and vaginal delivery mother-baby pairs. The C-section group exhibited a higher number of resistance genes associated with beta-lactam antibiotics as compared to the vaginal delivery group. These findings suggest a potential difference in the antibiotic resistance profiles between the two delivery modes.

Ultimately, the results of the end point PCR emphasise how critical it is to combat antibiotic resistance in both vaginal and C-section delivery methods. Healthcare professionals can decide on antibiotic therapy and strategies to stop the spread of resistant strains by having an in-depth understanding of the incidence and patterns of antibiotic resistance.

The patterns of antibiotic-resistant genes differ, which further necessitates ongoing monitoring and tracking of antibiotic resistance in both vaginal and C-section cases. In order to reduce the emergence of antibiotic resistance, it is vital to implement appropriate infection control strategies promoting careful consumption of antibiotics. In order to understand the underlying processes causing the variations in antibiotic resistance profiles between the two delivery modes, further research is required. It is

essential to explore the gut microbiota of mothers and other factors in order to understand the spread and uptake of antibiotic resistance genes, ultimately helping in the creation of focused therapies.

Future perspectives

Long-term follow-up: Conduct longitudinal studies to monitor changes in the gut microbiota of mother-baby pairs beyond the early postnatal period. This would shed light on the stability and evolution of the gut microbiota throughout time, as well as the possible consequences for health outcomes.

Functional Analysis: Investigate the functional properties of the discovered aerobic Gram-negative bacteria. Investigate their metabolic pathways, interactions with the host, and potential effects on host health or disease.

Microbial interactions: Investigate how aerobic Gram-negative bacteria interact with the rest of the gut microbiota, including both commensal and pathogenic species. Understanding these interactions can help us understand the dynamics of microbial populations and their effects on health.

Therapeutic Applications: Targeted therapy development may be aided by the findings of the study on antibiotic-resistant genes. Researchers can investigate methods to counteract antibiotic resistance and create alternate treatment modalities by determining the frequency and distribution of resistance genes.

Comparative Studies: Research examining populations and geographical areas can shed light on the ways that environmental, genetic, and cultural variables affect the gut microbiota. Finding similarities and differences in the gut microbiota of mother-baby pairs from different populations can help us gain a deeper understanding of microbial ecosystems.

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