A Comparative Assessment of Bacterial Load and Other High-Risk Factors of Necrotizing Enterocolitis among Neonates in Bangladesh

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A thesis submitted to the School of Life Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing a degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

The thesis/project titled “A Comparative Assessment of Bacterial Load and Other High-Risk Factors of Necrotizing Enterocolitis among Neonates in Bangladesh” submitted by Nusrat Jahan Shanta (22326053) and Ummay Sumaiya Tabassum (22326050), Fall 2026 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of B.Sc. in Microbiology in January, 2026.

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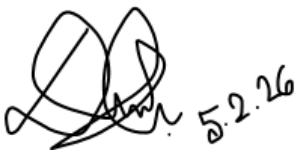

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Ethics Statement

The study was conducted following the Helsinki Declaration and subsequent revisions. The plagiarism check showed an overall similarity index of 13% for this thesis report.

Abstract

Background:

Necrotizing enterocolitis (NEC) is a serious inflammatory disease of the gastrointestinal tract that mainly affects preterm, low birth weighted neonates and remains an important cause of neonatal morbidity and mortality. In Bangladesh, evidence describing both intestinal bacterial burden and clinical high risk factors in NEC is still limited. This case-control study assessed bacterial load, different bacterial detection, and high risk factors among neonates in Bangladesh.

Methods:

A gender and gestational age matched case-control study was conducted at Ad-din Medical College Hospital, Bangladesh. The study population consisted of 66 preterm neonates (gestational age 28 to 36 weeks, birth weight up to 1.5 kg), including 33 NEC cases and 33 non-NEC controls. NEC cases were identified during sample collection based on key clinical features, including a positive occult blood test (OBT), abdominal distension, and the presence of bubble gas. Stool specimens were cultured on selective and differential media to measure bacterial load (CFU/ml) and to identify presumptive organisms based on colony morphology. PCR was performed to identify *Escherichia coli* and *Klebsiella pneumoniae*; PCR for other suspected organisms could not be performed due to unavailability of primers. To assess the significance of crude and adjusted odds ratio (aOR) of clinical relevance, Fisher's test and Chi-square test were applied.

Results:

Compared with NEC cases, controls showed a higher overall bacterial load, with a mean load of 1.25×10^7 CFU/ml in NEC and 2.21×10^7 CFU/ml in controls, representing an approximately 1.8 times higher load in the control group. However, PCR based detection identified *E. coli* and *K. pneumoniae* more frequently in NEC cases than in non-NEC. Growth on selective media suggested organisms such as *Staphylococcus epidermidis, Staphylococcus saprophyticus,* and *Pseudomonas aeruginosa*, along with several unidentified isolates. Several factors were analyzed including congenital heart disease, cardiogenic shock sepsis, formula feeding, breastfeeding, seizures and blood transfusion, only both type feeding demonstrated statistically significant difference between two groups (aOR: 0.8254; 95% CI: 0.2280-2.719; $p < 0.05$). Moreover, multidrug resistance was common in *K. pneumoniae* and *E. coli* isolates, and *E. coli* showed higher antimicrobial resistance.

Conclusion:

Our findings provide insight into clinical and microbiological parameters that may be useful for characterizing NEC. Because NEC is a complex inflammatory condition, further studies examining how multiple risk factors interact are needed to help lower the risk and impact of this devastating disease.

Keywords: *Necrotizing enterocolitis (NEC), occult blood test (OBT), bacterial load, PCR, bubble gas*

Dedication

We would like to keep all the neonates affected by NEC, especially those currently in the NICU, and their families in our thoughts and prayers. May Allah (SWT) bless these babies with healing and protection, and grant their parents patience and strength during this challenging time.

Acknowledgment

We would like to sincerely thank our supervisor, Dr. Nadia Sultana Deen, Associate Professor & Chairperson of Department of Microbiology, School of Life Sciences, BRAC University, for her continuous guidance, constructive feedback, and encouragement throughout this thesis. Her patience and supportive approach helped us navigate challenges with confidence. She consistently inspired us to think critically, refine our work, and stay motivated, and she always welcomed our ideas and results with genuine interest.

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List of Acronyms

CDC	Centers for Disease Control and Prevention
CFU	Colony-Forming Unit
CHD	Congenital Heart Disease
GDM	Gestational Diabetes Mellitus
LBW	Low Birth Weight
NEC	Necrotizing Enterocolitis
NICU	Neonatal Intensive Care Unit
OBT	Occult Blood Test
SGA	Small for Gestational Age
TLR-4	Toll Like Receptor-4
VLBW	Very Low Birth Weight

Chapter 1 Introduction

1.1 Background

Necrotizing enterocolitis (NEC) is a severe gastrointestinal condition that primarily affects neonates with very low birth weight (VLBW) and remains a major cause of morbidity and mortality in this vulnerable population (Patel & Shah, 2012). Premature infants are especially at risk due to the immaturity of their intestinal, immune, and vascular systems, which makes their intestines more susceptible to inflammation and injury. Several clinical factors, such as the timing and type of feeding, antibiotic exposure, and delayed intestinal adaptation, can further increase the likelihood of developing NEC (Xiang et al., 2022). The condition is difficult to diagnose early because its symptoms often resemble those of sepsis and other neonatal infections, which can delay intervention and worsen outcomes (Raturi & Chandran, 2024). Despite improvements in neonatal care, the incidence of NEC continues to be a significant concern, highlighting the need for better understanding of its underlying causes and risk factors (Shlomai et al., 2025).

Emerging evidence suggests that the balance and overall load of gut microbes play an important role in the development of NEC, influencing how the infant's intestines respond to stress and inflammation. Variations in microbial colonization, combined with other clinical risk factors, may contribute to the onset and severity of the disease (Thänert et al., 2020). In many regions, including Bangladesh, there is limited data on how these microbial and clinical factors interact in neonates with very low birth weight. This study aims to observe the relationship between microbial load and high-risk clinical factors, in order to identify patterns that may be associated with NEC development. Understanding these interactions can provide valuable insights for early detection, risk assessment, and potential preventive strategies, ultimately helping to improve outcomes for vulnerable neonates.

1.2 Pathophysiology of NEC

The specific mechanisms behind NEC remain unclear, even though it is widely accepted as a complex disease. (Singh et al., 2023) The pathophysiology of NEC refers to inflammation in the intestine allowing bacteria to invade, which lead to cell injury, cell death, and tissue necrosis. As the disease progresses, it may cause holes in the intestine, which can result in peritonitis, sepsis, and even death (Ginglen & Butki, 2023). The symptoms are not specific and can include feeding problems, vomiting, abdominal distension and immature intestine.

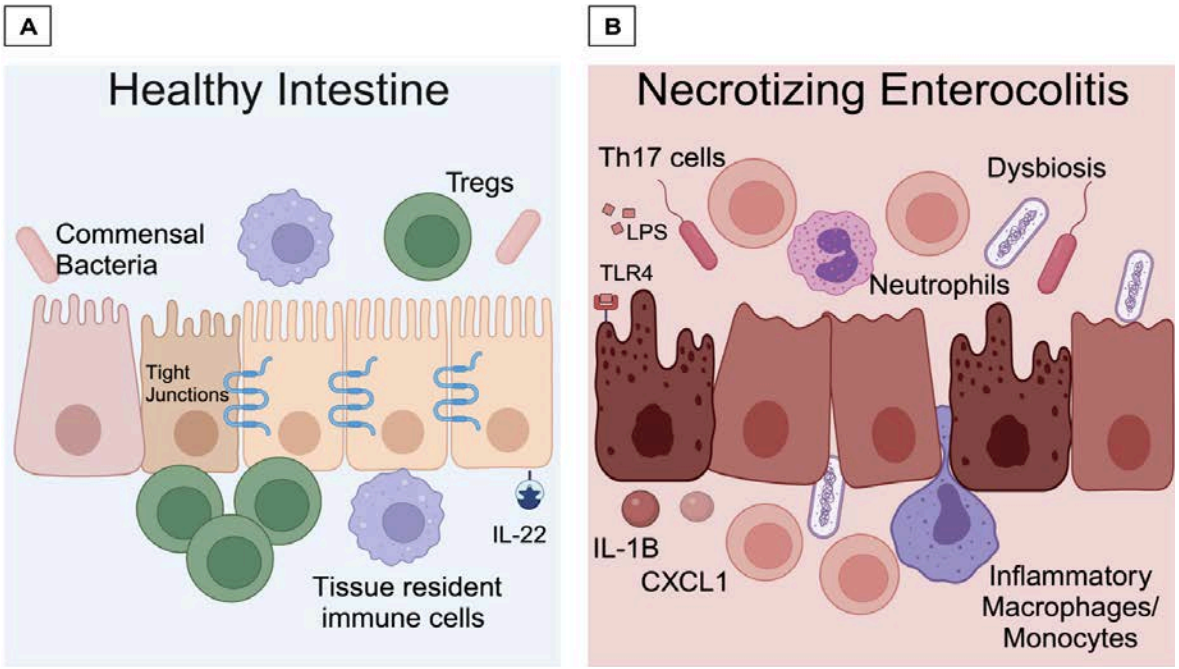

Figure 1.1: Differences between Healthy vs. NEC Intestine (A) Healthy Intestine vs. (B) NEC: Barrier and Immune Changes (Frazer et al., 2024).

Figure 1.1 shows how the intestine normally maintains balance, and how that balance breaks down in NEC. **Figure 1.1 (A)** illustrates the healthy gut, the lining of the intestine forms a strong barrier. Tight junctions seal the spaces between epithelial cells, which helps keep commensal bacteria in the gut lumen where they belong. The immune system stays calm and well-regulated, largely through regulatory T cells and other resident immune cells that support tolerance. IL-22 helps maintain this steady state by strengthening the epithelium and promoting repair (Frazer et al., 2024). On the other hand, **figure 1.1 (B)** illustrates the NEC gut microbiome which shifts toward dysbiosis, increasing exposure to inflammatory bacterial components such as liposaccharide (LPS). These signals activate TLR4 on the intestinal epithelium, placing stress on the lining and weakening the barrier. Once the barrier is compromised, inflammation escalates, Th17 responses increase, neutrophils are recruited, and inflammatory macrophages and monocytes accumulate. Cytokines and chemokines such as IL-1β and CXCL1 further amplify immune cell recruitment and inflammation, driving ongoing epithelial injury, leakiness, and the self-perpetuating inflammatory cycle typical of NEC (Frazer et al., 2024).

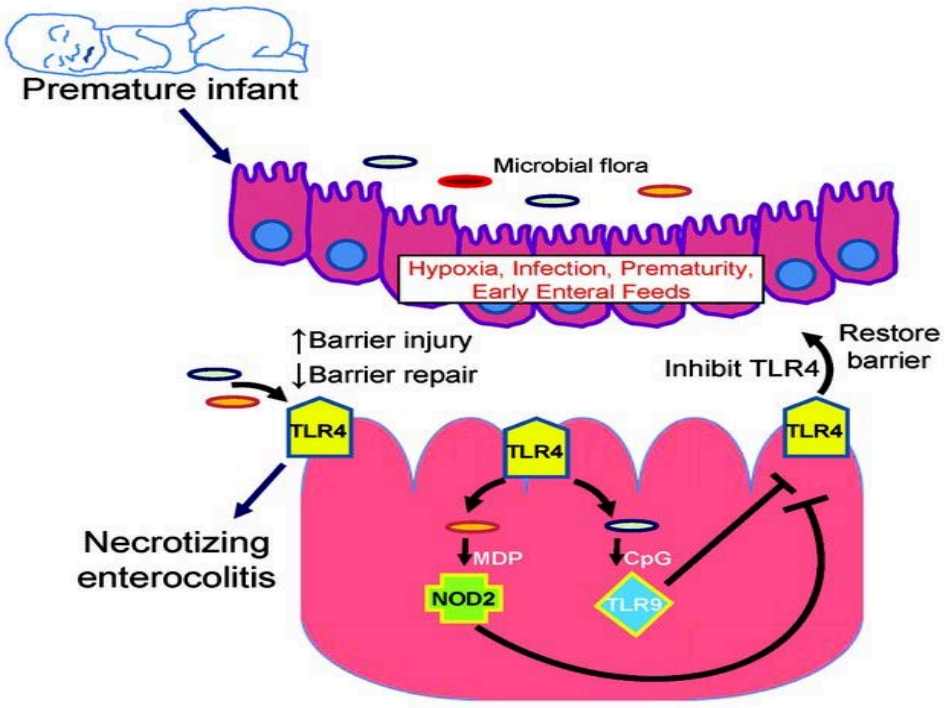

Figure 1.2: Pathophysiology of NEC (Afrazi et al., 2010).

This figure explains NEC as a consequence of an immature intestinal barrier and an exaggerated innate immune response to gut bacteria in premature infants. In prematurity, the intestinal lining is fragile and still developing, while microbial colonization is beginning. Stressors such as hypoxia, infection, early enteral feeding, and prematurity itself can disrupt the normal balance of gut flora and place additional inflammatory stress on the epithelium (Hackam & Caplan, 2017). A key mechanism shown is heightened activation of toll-like receptor 4 (TLR4) on enterocytes in response to microbial products. Excess TLR4 signaling increases epithelial injury and impairs normal barrier repair, leading to loss of mucosal integrity and increased permeability. This breakdown promotes inflammation and bacterial translocation, driving the progression toward necrotizing enterocolitis (Ishiyama et al., 2025). The diagram also highlights protective pathways that counterbalance this response. Nucleotide-binding oligomerization domain containing protein (NOD2) activation by muramyl dipeptide (MDP) and TLR9 activation by CpG DNA inhibit TLR4 signaling, which supports restoration of barrier function. Overall, the **figure 1.2** presents NEC as an overactive TLR4-mediated inflammatory response, moderated in part by inhibitory signals from NOD2 and TLR9 (Van Heel, 2005). The main risk factors of NEC are described as follow:

1. **Immature Intestine:** Due to immature gastrointestinal mechanisms, including immature motility, digestion , absorption, immunological defenses, and barrier function, preterm infants are more vulnerable to intestinal damage (Snyder & Hunter, 2023). For example, low stomach acid secretion, particularly in neonatals, might result in NEC, specifically when H2 blockers further suppress it (Nugent et al., 2024). Additionally, preterm neonates exhibit a higher level of inflammation in response to luminal microbial stimuli, which changes the intestinal protective barriers. This is also related to reduced expression of regulatory proteins like IκB, which affect inflammatory pathways, and increase the expression of toll-like receptor 4 (TLR4) (Kuzmich et al., 2017). In addition, neonates with NEC exhibit higher serum concentrations of various cytokines and chemokines that attract inflammatory cells compared to healthy preterm neonates (Hunter & De Plaen, 2014). However, one of the cytokines found at increased levels is interleukin-8, which is produced by epithelial cells, that promotes the migration and activation of neutrophils in inflammatory sites (Godaly et al., 1997). This activity also contributes to the tissue damage through necrosis and improves the acute phase protein production in the gut of infants.

2. **Improper Microbial Colonization:** Another significant factor for NEC development is abnormal microbial colonization (Thänert et al., 2021). The presence of unusual bacterial species and decline in a diversified gut microbiota that happens due to an extended antibiotic treatment course are the two causes of this condition (Zhu et al., 2022). However, only abnormal microbial colonization is closely related to the pathogenesis of this disease. In general, the presence of diverse gut microbiota protects from nosocomial infections but NEC patients often lack this varied diversity (Beharry et al., 2023). Due to unstable developmental immaturity in regulatory systems and reduced expression of IκB, an increased inflammatory response has been shown by the preterm infants' enterocytes (De Plaen, 2013). So, this immature response is thought to be a significant factor contributing to the pathogenesis of NEC along with modified microbiota.

3. **Hypoxia-Ischemia:** Hypoxia-ischemia was identified as a major cause for the pathogenesis of NEC but it is rather one of the several contributing factors (Tagin et al., 2012). The alteration of microvascular conditions influences the development of

NEC by regulating the production of vascular mediators such as nitric oxide and endothelin, that play a role in the downstream pathogenic cascade that drives (Nowicki, 2005). The pathophysiology of NEC may also be influenced by factors like hypoxia-ischemia or blood transfusions, both of which can affect the intestinal blood flow (Kaplina et al., 2023).

1.3 Diagnosis of NEC

The lack of an accurate diagnostic method is a significant obstacle in the supervision and treatment of NEC. (J. H. Kim et al., 2020) Bell's strategy is still the only diagnosis method which is used to diagnose NEC until today. The first classification of NEC was put forward by Bell and colleagues in 1978 (Patel et al., 2020). To distinguish infants by the severity of their illnesses, provide treatment, and facilitate reliable comparisons of NEC supervision, the staging system of Bell consisted of features that were used to manage neonates for either of the three stages of NEC (**Table 1.1 and 1.2**).

However, this strategy was insufficient to determine the exact pathophysiology of NEC. Bell's strategies were updated and divided into 3 to 6 classes in 1986 (Patel et al., 2020). The criterion of bright red blood from the rectum (Stage IB) is the most recent staging approach used to distinguish between the neonates with Bell stage I and those without this finding (Stage IA). Besides, Stages IIA and IIB made it possible to distinguish between neonates who were slightly sick (Stage IIA) and those who were moderately sick (Stage IIB) due to portal venous gas or ascites. Lastly, in contrast to stage IIIA, the stage IIIB detected infants with pneumoperitoneum infection. As a result, the absence of precise and reliable medical biomarkers for diagnosis has become another challenge (**Table 1.1 and Table 1.2**). Thus, without a pathological gastrointestinal specimen the diagnosis is difficult due to unclear clinical criteria such as radiographic idiosyncrasies (detect pneumatosis in the intestinal wall versus bubbles in feces) and the absence of specific biological markers for the diagnosis of NEC (Van Druten et al., 2019).

1.4 Risk Factors of NEC

NEC is a life-threatening disease in premature infants in the Neonatal Intensive Care Unit due to gut dysbiosis. The pathophysiology of NEC includes various risk factors which are as follows:

1.4.1 Maternal Factors

The neonates are in contact with the mother from the embryo stage till delivery for which maternal factor is crucial for NEC development. The maternal history includes gestational diabetes mellitus (GDM), eclampsia, hypothyroidism, UTI, anemia, intrauterine growth restriction (IUGR), delivery type and fetal hypoxia that play a significant role in NEC.

Table 1.1: Comparison of Risk Group, Exclusion Criteria, and Systemic Signs across NEC definition

Bell's Criterion															
Variable	Bell Staging			Modified Bell Staging						**UK**	**VON**	**CDC**	**2/3**	**ST**	**INC**
	I	**II**	**III**	**IA**	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**						
Risk grouping										+			+		+
GA															
Postnatal or PMA													+	+	+
Gender														+	
Ethnicity														+	
Exclusion															
SIP										+	+		+		+
Congenital anomaly													+		+
Fed <80ml/kg/day													+		
A≥ 36 weeks													+		+
Systemic signs															
Temp. instability	+	+	+	+	+	+	+	+	+						
Apnea	+	+	+	+	+	+	+	+	+						
Bradycardia	+	+	+	+	+	+	+	+	+						
Lethargy	+	+	+	+	+	+	+	+	+						
Acidosis (mild)								+	+	+				+	
Thrombocytopenia								+	+	+			+	+	+
Hypotension/Shock									+	+					
Acidosis									+	+				+	
DIC									+	+					+
Neutropenia									+	+					
Ventilated														+	

INC: International Neonatal Consortium; CDC: Centers for Disease Prevention and Control; VON: Vermont Oxford Network; NEC: Necrotizing Enterocolitis; GA: Gestational Age

Table 1.2: Comparison of intestinal signs and radiologic findings across NEC definitions

Bell's Criterion															
Variable	Bell Staging			Modified Bell Staging						**UK**	**VON**	**CDC**	**2/3**	**ST**	**INC**
	I	**II**	**III**	**IA**	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**						
Poor Feeding Intolerance	+	+	+												
Emesis	+	+	+	+	+	+	+	+	+		+	+			
Pre-gavage residuals	+	+	+	+	+	+	+	+	+	+					
Bilious aspirates	+	+	+							+	+	+			
Abdominal distention (mild)	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Marked distention		+	+					+	+						
Guaiac-positive stool	+	+	+	+	+	+	+	+	+						
Rectal bleeding(occult)	+	+	+		+	+	+	+	+	+	+	+	+		+
Marked hemorrhage			+												
Absent bowel sounds						+	+	+	+				+		+
Abdominal tenderness						+	+	+	+	+					
Marked tenderness								+	+						
Generalized peritonitis								+	+						
Abdominal cellulitis							+	+	+						
Right low quadrant							+	+	+						
Abdominal discoloration				+	+					+				+	
Radiological findings								+	+	+			+	+	+
Normal									+	+					
Ileus	+	+	+	+	+	+	+	+	+				+		
Pneumatosis		+	+			+	+	+	+	+	+	+	+	+	+
Portal Venous gas		+	+			+	+	+	+	+	+	+	+	+	+
Ascites							+	+	+						
Pneumoperit			+						+	+	+	+			

oneum															
Fixed loop		+	+							+					
Small bowel separation		+	+												

INC: International Neonatal Consortium; CDC: Centers for Disease Prevention and Control; VON: Vermont Oxford Network; NEC: Necrotizing Enterocolitis; GA: Gestational Age

1. **Intrauterine Growth Restriction (IUGR):** The small for age (SGA) neonates are associated with a condition called intrauterine growth restriction which increases the risk of NEC. It is a maternal factor that will affect the fetus. Besides, high blood pressure, anemia, smoking, kidney disease and diabetes can disrupt the nutrition and oxygen to the fetus which gradually results in SGA infants (Blue et al., 2021).

2. **Types of Delivery Methods**: Most of the preterm babies are delivered through cesarean section rather than vaginally at present. The connection between the type of delivery with NEC development is still unclear. Besides, the babies delivered vaginally come in contact with the mother's feces and get exposed to the microbial community. However, in case of cesarean delivery, the baby is more prone to be affected by nosocomial infection (Weng & Walker, 2013).

3. **Gestational Diabetes Mellitus:** The high blood sugar level of the mother interrupts the blood circulation of the neonate due to connection for fetal nutrition**.** This can lead to necrosis and inflammation of the intestinal mucosa. due to which the harmful bacteria colonization will be easier.

4. **Premature Rupture of Membranes:** The premature delivery of the mother is a major risk factor which can lead to the development of NEC (Dayal et al., 2024). The delivery of the baby before 37 weeks of gestation refers to a condition PROM. These early delivered babies are at high health risk for incomplete development.

1.4.2 Infant-Related Factors

1. **Preterm:** The babies delivered before 37 weeks of gestation and birthweight below 1.5kg are referred to as preterm babies. They are also classified as very preterm, moderate preterm and low preterm. Due to an underdeveloped intestine and nervous system, they are more vulnerable to excess bacterial colonization, resulting in NEC.

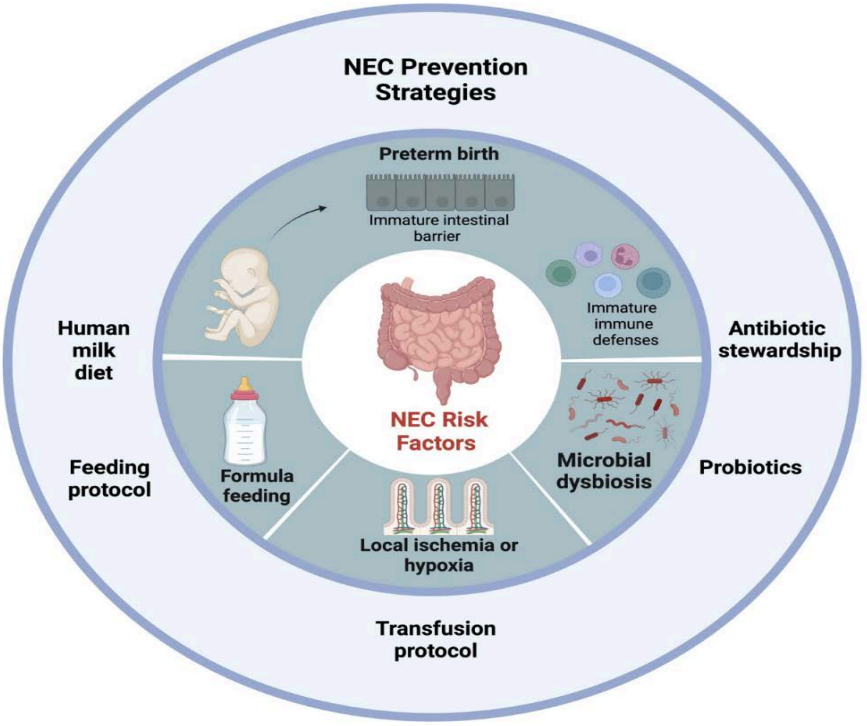

Figure 1.3 : Risk factors of NEC (Colarelli et al., 2024).

2. **Low Birth Weight:** The infants born with birth weight below 2.5kg refer to low birth weight because of immature delivery or fetal growth restriction.The intestines of the neonates are very immature causing gut dysbiosis for being easily exposed to pathogenic microorganisms.

3. **Preterm Birth weight:** The babies born below 1.5kg birth weight are considered as preterm babies. The preterm birth is also further classified as extreme- preterm (less than 28 weeks), very- preterm (28 weeks to less than 32 weeks) and moderate to late-preterm birth (32 weeks to 37 weeks) (World Health Organization: WHO, 2023). The extremely preterm babies are at high risk of NEC development because of underdeveloped organs.

4. **Blood Transfusion**: The blood transfusion has an association with NEC development among the infants with low birth weight and gestational age (Cunningham et al., 2017). TNF-α, IL-6, and Paroxysmal Atrial Fibrillation (PAF) are the inflammatory mediators which are produced during the blood process and storage of blood cells are related to the pathogenesis of NEC (Nasab et al., 2025). Besides, free hemoglobin, stored RBC, remaining WBC and the cell membrane fragments of RBC are also related to NEC (Salem & Patel, 2023). The RBC undergoes some pathological modifications which include reduced deformability, increased oxygen affinity and reduced level of nitric oxide, that affect the vasodilation during storage.

5. **Small for Gestational Age (SGA)**: Some risk factors like neonatal asphyxia, respiratory distress, hypoglycemia, brain injury etc. are observed in the SGA infants. These infants are more likely to be associated with a growth condition called IUGR (Philadelphia, n.d.).

6. **Occult Blood Test (OBT):** The Occult Blood Test is a simple bedside investigation used in neonates with suspected NEC. Because NEC causes inflammation and ischemic damage to the intestinal wall, mucosal injury and bleeding are common, leading to the presence of occult blood in stool. OBT detects small amounts of blood that are not visible on gross examination (Philadelphia, n.d.). In NEC, a positive OBT suggests intestinal mucosal injury and impaired gut integrity and may help raise early suspicion of the disease. Studies have shown that neonates who develop NEC often demonstrate positive OBT results before definitive clinical or radiological findings appear. However, OBT is not specific for NEC, as positive results may also occur in other neonatal conditions such as swallowed maternal blood, anal fissures, sepsis, or milk protein intolerance (Ginglen & Butki, 2023b). Therefore, OBT should not be used alone to confirm NEC but should be interpreted in conjunction with clinical features and radiological evidence. Despite these limitations, OBT remains a useful supportive tool due to its low cost, ease of use, and non-invasive nature, particularly in resource-limited neonatal intensive care settings (Philadelphia, n.d.).

7. **Radiological Evidence of Gas:** Radiological identification of abnormal gas within the gastrointestinal tract is the most definitive method for confirming necrotizing enterocolitis. The hallmark finding is pneumatosis intestinalis, also known as “bubble

gas," which represents gas within the bowel wall caused by bacterial gas penetrating a damaged intestinal mucosa (Weerakkody et al., 2009). On abdominal radiographs, this appears as linear or cystic lucencies along the bowel wall and is highly specific for NEC. In more severe cases, gas may extend into the portal venous system, indicating advanced disease and a higher risk of bowel necrosis or perforation, while additional findings may include bowel dilatation, fixed loops, or pneumoperitoneum (*The Radiology Assistant: Necrotizing Enterocolitis*, n.d.). Early imaging findings can be nonspecific but the presence of pneumatosis intestinalis strongly supports the diagnosis of NEC, making radiological evidence the cornerstone of NEC confirmation (Gao & McKinney, 2020).

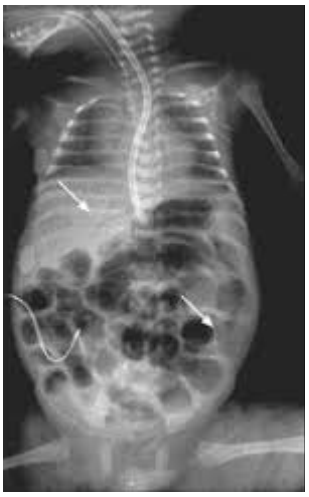

Figure 1.4 : Abdominal X-ray showing Gas Bubbles in a NEC Baby's Intestine (Neu & Walker, 2011).

8. **Other Factors:** The other risk factors of NEC include presumed sepsis, congenital heart disease (CHD), seizure, vitamin D insufficiency, twin pregnancy, consanguineous parents and pneumonia. The intestinal mucosa of the infant is directly or indirectly affected when the body produces various inflammatory transmitters. Besides, intestinal epithelial cell necrosis can occur due to the endotoxin and other products secreted by the bacteria (S. Patel et al., 2010). In pneumonia or respiratory distress, infants may develop hypoxia, disrupting blood flow and potentially leading to brain injury (*Postoperative Outcomes, and Growth and Brain Injury Outcomes in Spontaneous Intestinal Perforation Vs Surgical Necrotizing Enterocolitis in Preterm Infants*, 2023).

1.5 Epidemiology of NEC

NEC accounts for 10% newborn mortality across the globe (Sha et al., 2025). In 2019, Centers for Disease Control and Prevention's (CDC) National Center for Health Statistics (NCHS) stated that among 10 dominant causes of neonate death, NEC is one of the major causes (*Products - Data Briefs - Number 395 - December 2020*, n.d.). This life threatening emergency generally affects neonates in the second to third weeks of their life. Amid contributing factors to cause this fatal disease, premature birth, VLBW and formula feeding have been determined as leading risk factors. Alongside, genetic factors can have a vital role in progressing this disease. Moreover, among per thousand births of live infants, incidence of NEC differs globally from 0.3 to 2.4. Approximately 7 out of 10 cases arise in preterm children who were brought into this world earlier than usual gestational age. Furthermore, the death incidence ranges from 10% to 50% around the world. In spite of that, the mortality rate reaches 100%, consisting of perforation, sepsis and peritonitis in the most critical instances. Although NEC mostly occurs in preterm babies, it is also reported in full term babies (*Maternal and Foetal Risk Factor and Complication With Immediate Outcome During Hospital Stay of Very Low Birth Weight Babies*, 2012). In the United States, the prevalence of NEC spans from 1 to 7.7% admission in the NICU and among them 90% of the admitted cases are preterm babies. Moreover, while documenting the prominent pathogenic risk factors in these babies, mucosal damage and colonization of bacteria were found associated with NEC (Kosloske, 1994). A quantitative analysis was conducted with several studies where from a hospital based sample of Belgium, it was known that the morbidity rate of NEC is 16.23%, the national registry data from 2005-2013, showed that the occurrence of NEC in Finland is 16.58% among premature babies and from three tertiary centers of Romania, it was observed that about 16.6% preterm infants are sufferers of NEC (Alsaied et al., 2020). Besides, if any NEC victims survive, they have to face lifelong health complications throughout their lives, such as- prolonged gastrointestinal problems, psychological challenges and growth-related or neurological difficulties (Canvasser et al., 2022). Over the past few years, progression in feeding habits, breast milk feeding practices and complete supervision by doctors of maternal health during pregnancy has played a significant role in declining NEC (Lawrence, 2010).

1.6 Study Gap

A significant high rate of low-birth-weight neonates (about 23 neonates among 1000) also indicates that the rate of NEC cases may remain high in Bangladesh (Baqui et al., 2013).

Additionally, the bacterial and other risk factors associated with NEC in Bangladeshi neonates are also unknown. However, there is scarcity of NEC data in Bangladesh.

Given this background, we hypothesize that the incidence of NEC would be high in Bangladesh, and the bacterial and other risk factors of NEC might shed light on the purpose of this study is to identify risk factors to perceive and diminish the effect of NEC on neonates. Until now, in Bangladesh, no case-control study has been conducted to inspect the fatality rate, risk factors and prevalence of NEC, establishing it as a pathbreaking initiative in the country.

1.7 Aims of the Study

The key aim of this study was to investigate the gut microbial load and other risk factors found in suspected NEC infants in Bangladesh. The precise objectives are:

1. To identify the risk factors observed in NEC infants.
2. To assess the intestinal gut microbial load between NEC and non-NEC neonates.
3. To isolate and identify the bacterial species present in both NEC and non-NEC groups to identify the distinctions in bacterial composition.
4. To evaluate the pattern of antimicrobial resistance of isolated bacteria from both groups.

Chapter 2 Methods and Materials

2.1 Study Design

The study was designed as a case-control study. The study was conducted in Ad-Din Medical College and Hospital, Dhaka.

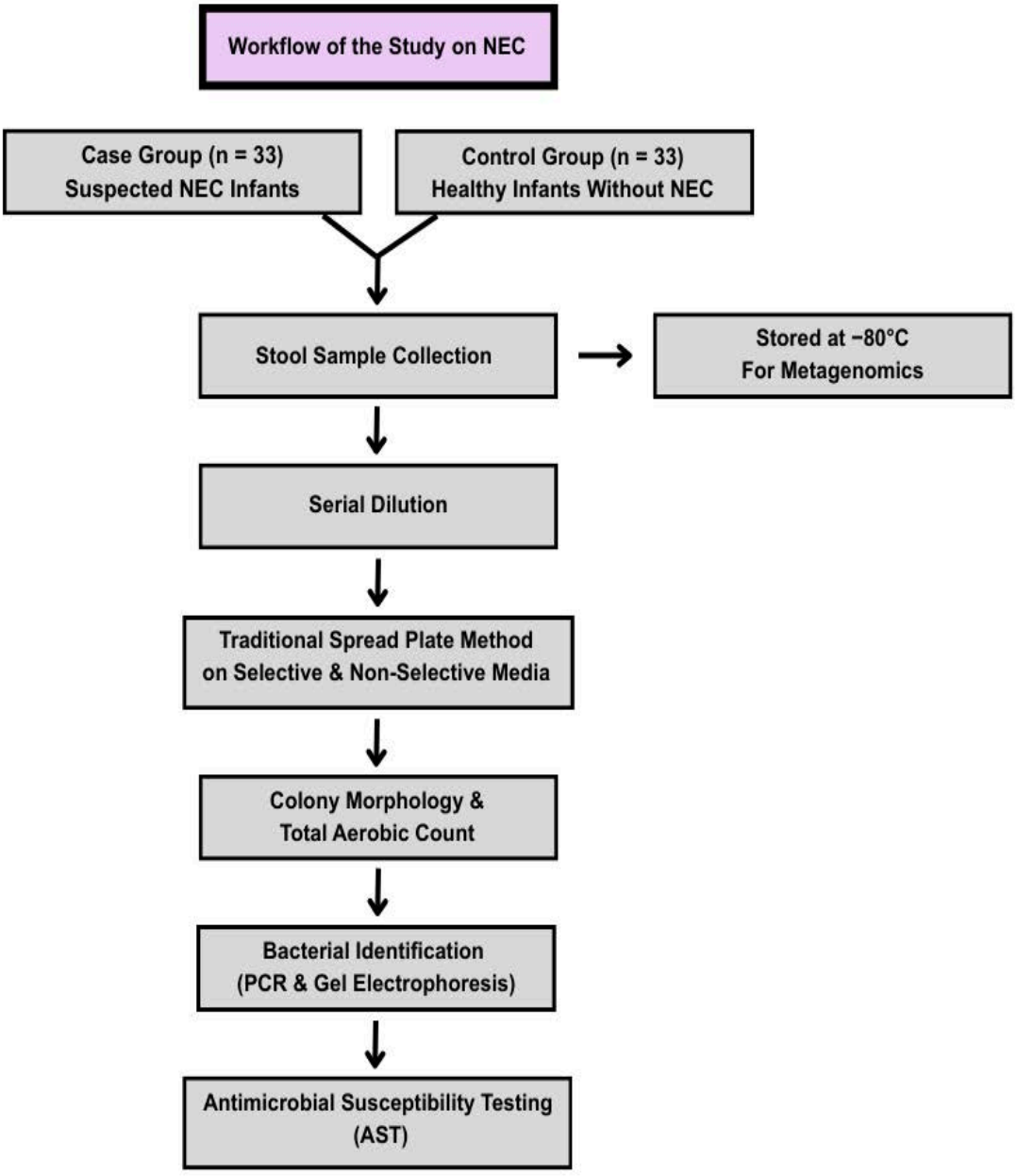

Figure 2.1 : Schematic Illustration of the Study Design.

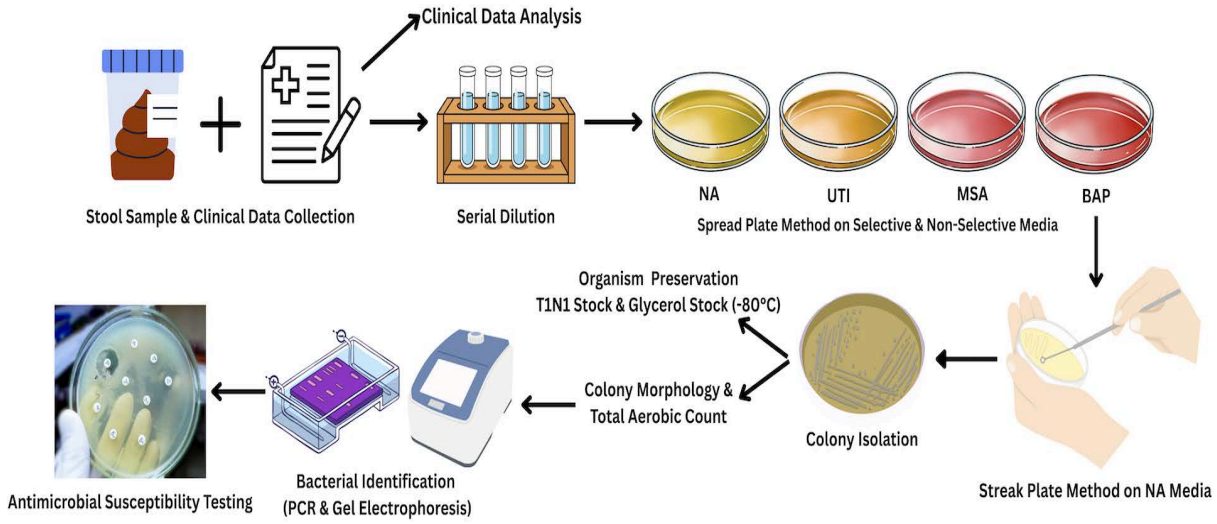

Figure 2.2 : Schematic Illustration of the Workflow.

2.2 Selection of the Suspected NEC Case and Non-NEC Controls:

Suspected NEC Case: A total number of 33 suspected NEC cases with significant clinical findings, such as- distended abdomen, low birth weight, premature infant, vomiting, feeding intolerance were selected as our case for the study, and

Non-NEC Control: A total number of 33 gender and gestational age-matched healthy neonates without any NEC symptoms were chosen as controls from the corresponding hospital.

Table 2.1 : Inclusion and Exclusion Criteria

Participant	**Inclusion Criteria**	**Exclusion Criteria**
Cases (Suspected NEC)	a) Infants admitted in the NICU with NEC symptoms b) Gestational age between 28 to 36 weeks c) Birth weight ≤ 1.5 kg d) OBT (Occult Blood Test) positive e) Radiology: bubble gas present	a) Healthy infants outside the NICU
Control (Non-NEC)	a) Healthy infants without NEC symptoms b) Gestational age matched to cases c) Gender matched to cases d) Birth weight ≤ 1.5 kg e) OBT negative f) Radiology: bubble gas absent	a) Healthy infants outside the NICU

2.3 Questionnaire

A structured questionnaire was created to gather relevant clinical and demographic data from both suspected NEC and non-NEC groups. It was designed by focusing on related risk factors of NEC, which includes sections on demographic data, clinical data and maternal data.

1. **Demographic Data:** This section of the questionnaire includes demographic information, such as- parent's name, mother's blood group, age, occupation etc.

2. **Clinical Data:** This part comprises the clinical history of the baby, for instance, the birth weight, gender, gestational age, blood group, any prospective symptoms related to NEC, such as- abdominal distention, fever, vomiting,feeding intolerance, any history of surgery or blood transfusion etc. Moreover, the history of antibiotics side by side with any other supplements that have been used to treat the baby were also documented.

3. **Maternal History:** In this portion, the mother's history was recorded focusing on the type of delivery, if the mother has taken any antibiotic during the time of pregnancy and after delivery, any complications that have been observed recently.

2.4 Consent and Ethical Issues

The study was approved by the proper authority from the hospital. Moreover, the purpose and protocol of the study was fully aware by the parents of the participants and they gave verbal consent for it. The questionnaire was filled up with the permission and in the presence of the attending healthcare professionals directly from the participant's report and mother's history was also taken using the questionnaire.

2.5 Sample Collection and Processing

2.5.1 Stool Sample

A sterile swab was used to collect approximately 0.2 grams of stool from the infants in the blue-capped plastic container. It is stored at -80°C until further processing. Before sample processing the stool sample needs to be thawed in crushed ice for 10 minutes.

Figure 2.3 : Collected Stool Sample.

2.5.2 Serial Dilution

To perform serial dilution, 0.2 gm of stool sample was diluted with 5ml saline solution and vortexed to mix uniformly. After that, 7 more test tubes were taken in which 9ml saline solution was added and labelled as 10^{-0},10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Then, 1ml was transferred from the vortexed main sample to the 7 test tubes simultaneously and each vortexed before transferring. Lastly, 1 ml was discarded from the last test tube (10^{-6}) and 0.1µL of diluted sample from each dilution was plated on the selected media by spreading method and incubated under required conditions. This method is used for CFU count by the

following formula:

$$\text{CFU/mL} = \frac{\text{Number of colonies x Dilution factor}}{\text{Volume plated(mL)}}$$

TFTC (too few to count) when the colony ranges more than 300 and TNTC (too numerous to count) when colony is less than 30 (Libretexts, 2021).

2.5.3 Detection of isolated bacteria based on colony characteristics

Mannitol Salt Agar (MSA), HiCrome UTI agar, Nutrient Agar (NA), and Blood Agar (BA) were used for colony isolation and presumptive bacterial identification.

2.6 Molecular Identification of the Isolated Bacteria

2.6.1 DNA Extraction by the Boiling Method

TE buffer (Tris-EDTA buffer, 200 µL) was taken into a sterile microcentrifuge tube. Then, 1 loopful of a bacterial colony was added to the TE buffer and mixed thoroughly. The suspension was vortexed to ensure proper mixing of the bacterial cells with the buffer. The tube was then centrifuged at 13,000 rpm for 10 minutes to pellet the bacterial cells. After centrifugation, the supernatant was carefully discarded, and the cell pellet was retained. Again, 200 µL of fresh TE buffer was added to the pellet and mixed properly. The suspension was then placed in a boiling water bath at 100°C for 10 minutes to lyse the cells. After boiling, the sample was centrifuged at 10,000 rpm for 10 minutes to separate cell debris from the released DNA. Finally, the supernatant containing the extracted DNA was carefully collected and transferred to a new sterile microcentrifuge tube for downstream applications.

2.6.2 NanoDrop (Microvolume Spectrophotometry)

The NanoDrop model NanoEX Plus was switched on, and the appropriate nucleic acid measurement mode was selected. The optical pedestals were cleaned with nuclease-free water and wiped gently using lint-free tissue to remove any residue. Then, 2 µL of TE buffer were loaded onto the lower pedestal, and the instrument was blanked to calibrate the system. After blanking, the pedestals were cleaned again to remove the buffer. 1-2 µL of the extracted DNA sample was placed onto the lower pedestal. The upper pedestal was lowered carefully to form a liquid column between the two optical surfaces. The absorbance was measured automatically by the instrument, and the DNA concentration and purity ratios (A_{260}/A_{280} and A_{260}/A_{230})were recorded. After measurement, the sample was removed, and the

pedestals were cleaned thoroughly with nuclease-free water and lint-free tissue. The initial calibrating steps were repeated for all DNA samples. Following NanoDrop analysis, the extracted DNA samples were diluted to a final volume of 10 µL using TE buffer and were then used for downstream applications such as PCR.

2.6.3 Polymerase Chain Reaction (PCR):

Firstly, using a micropipette, 6.5 µL of PCR master mix was transferred into a sterile PCR tube. Then, 1 µL of forward primer and 1 µL of reverse primer were then added to the same tube. 2.5 µL of nuclease-free water was transferred into the PCR tube and 2 µL of the previously extracted DNA sample were added to the reaction mixture. The contents of the tube were mixed gently to ensure proper homogenization of all components. The PCR machine was then programmed according to the PCR cycling conditions specific to the target organism. The PCR tubes were placed into the thermal cycler, and the lid of the machine was closed securely. After completion of the PCR run, the tubes containing the amplified DNA products were stored appropriately for further analysis.

2.6.4 Gel Electrophoresis

At first, 1.5 grams of agarose was weighed and added to 100 mL of 1x TBE buffer in a conical flask. The mixture was heated until the agarose was completely dissolved and the solution became clear. The agarose solution was then allowed to cool slightly, after which 3-4 µL of ethidium bromide (EtBr) was added and mixed gently. The molten agarose was poured into a gel casting tray, and a gel comb was placed near the black electrode of the apparatus. The gel was left undisturbed until it solidified, after which the comb was carefully removed. The solidified gel was then placed into the electrophoresis chamber and immersed in a 1x running buffer (TBE or TAE). The PCR products were loaded into the wells in the following order: DNA ladder, positive control, negative control, sample DNA and lastly DNA ladder again. The lid of the electrophoresis apparatus was then closed securely. Electrophoresis was carried out at 100 volts for approximately 50 minutes. After about 40 minutes of electrophoresis, the DNA bands were observed under a UV illuminator.

2.7 Antibiotic Susceptibility Test (AST)

The Antibiotic Susceptibility Test (AST) was performed using the Kirby-Bauer disc diffusion method.

2.7.1. Preparation of the Inoculum

Before testing, the inoculum was standardized. A sterile loop was used to collect colonies from freshly sub-cultured nutrient agar plates, which were then inoculated into 10 mL of sterile sodium chloride (NaCl) solution in a test tube. The mixture was vortexed to create a uniform suspension. Turbidity was adjusted to match a 0.5 McFarland standard, representing approximately $1\text{-}1.5\times10^{8}$ CFU/mL. If the turbidity fell outside the standard range (optical density between 0.08-0.1), the concentration was corrected by either dilution or further addition of the bacterial suspension.

2.7.2. Inoculation of the MHA Plate

A sterile swab was dipped into the standardized inoculum and pressed against the inside wall of the test tube to remove excess liquid. The swab was then streaked evenly across the surface of the dried MHA plate in three different directions, rotating the plate approximately 60 degrees between each streak to ensure even distribution. After streaking, the plate was left partially open at room temperature for 3 to 5 minutes to allow for proper drying.

2.7.3. Placement of Antibiotic Discs

Sterile forceps were used to place the antibiotic discs onto the inoculated MHA plate. Each disc was gently pressed to ensure proper contact with the agar surface. One disc was placed at a time until all 12 antibiotics had been applied, following the specified arrangement. The plates were then incubated at 37°C for 24 hours. After incubation, the presence or absence of zones around the discs was observed to evaluate bacterial sensitivity or resistance to each antibiotic.

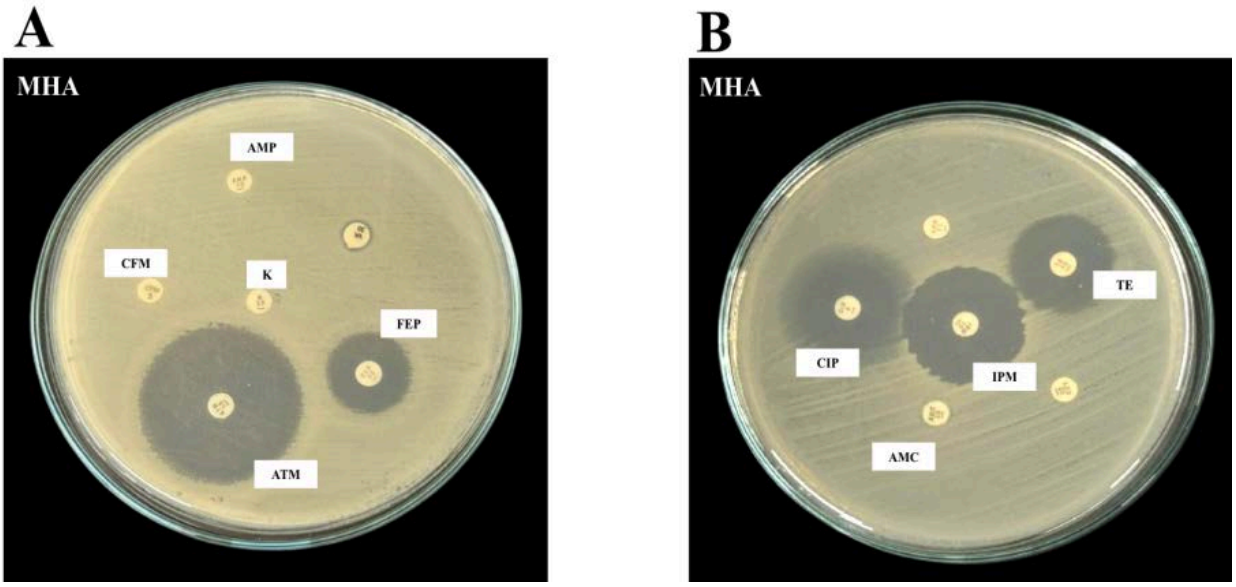

Figure 2.4: Placement of Antibiotic Discs.

Table 2.2 : List Of Antibiotic Disks, Their Group and Concentrations

Sl.	Antibiotics	Antibiotic Group	Disc Code	Disc Potency (µg)
1	Kanamycin	Aminoglycosides	K	30
2	Cefixime	Cephalosporins (3rd gen)	CFM	5
3	Aztreonam	Monobactams	ATM	30
4	Cefipime	Cephalosporins	FEP	5
5	Ampicillin	β- Lactam	AMP	10
6	Imipenem	Carbapenem	IMP	10
7	Tetracycline	Tetracycline	TE	30
8	Amoxicillin/Clavulanic Acid	Penicillin (Beta-lactamase inhibitor)	AMC	30
9	Ciprofloxacin	Fluoroquinolone	CIP	5

Chapter 3 Results

3.1 Demographic Data

A total of 66 preterm infants were included in the study, comprising 33 suspected NEC cases and 33 non-NEC controls. The suspected NEC case and non-NEC control groups were matched for gestational age and gender. The infants had gestational ages ranging from 28 to 36 weeks, and their birth weights ranged from 0.8 kg to 1.5 kg. All participants were selected from Ad-Din Hospital.

Table 3.1 : Demographic Data

Characteristics		**Suspected NEC**	**Non-NEC**
Gender	Male	17(51.5%)	17 (51.5%)
	Female	16(48.5%)	16(48.5%)
	Total	33(100%)	33(100%)
Weight (Kg)		1.28 (Average)	1.26 (Average)
Gestational Age (Weeks)		33.3 (Average)	33.2 (Average)

The table summarizes the demographic characteristics of the study groups (n = 33 each). In both the suspected NEC and non-NEC groups, 17 (51.5%) were male and 16 (48.5%) were female. The mean birth weight was 1.28 kg in the suspected NEC group and 1.26 kg in the non-NEC group. The mean gestational age was 33.3 weeks for suspected NEC and 33.2 weeks for non-NEC **(Table 3.1)**.

3.2 Clinical Data

Clinical characteristics and associated risk factors were recorded for both the suspected NEC and non-NEC groups.

Table 3.2 : Clinical Characteristics of Suspected NEC and Non-NEC Groups

Characteristics		**Suspected NEC**	**Non-NEC**
Methods of Feeding	Formula Feeding	6	11
	Breast Feeding	4	2
	Both	23	20
Feeding Difficulty		5	1
Premature		7 (21.2%)	8 (24.2%)
VLBW		12 (36.6%)	12 (36.6%)
LBW		14 (42.4%)	13 (39.4%)
Vitamin D Deficiency		13 (39.39%)	20 (60.61%)
Pneumonia		3 (9.09%)	4 (12.12%)
Vomiting		23 (70%)	19 (58%)
Blood Transfusion		12 (36.6%)	13 (39.4%)
One Half of a Pair of Twins		3 (9.09%)	3 (9.09%)
Consanguineous Parents		3 (9.09%)	2 (2.06%)
CHD		13 (39.4%)	11 (33.3%)
Sepsis		29 (87.88%)	24 (72.73%)
Cardiogenic Shock		1 (3.03%)	2 (6.06%)
Seizure		3 (9.09%)	5 (15.15%)
Number of Antibiotics Prescribed for each		4.18	3.12

This summarizes the clinical characteristics of the suspected NEC (n = 33) and non-NEC (n = 33) groups. Formula feeding was reported in 6 and 11 neonates, breast feeding in 4 and 2, and mixed feeding in 23 and 20 neonates, respectively. Feeding difficulty was recorded in 5 suspected NEC cases and 1 non-NEC case. Prematurity was observed in 7 (21.2%) suspected NEC and 8 (24.2%) non-NEC neonates. VLBW was present in 12 (36.6%) in both groups, and LBW in 14 (42.4%) and 13 (39.4%), respectively. Vitamin D deficiency was noted in 13 (39.39%) suspected NEC and 20 (60.61%) non-NEC neonates. Pneumonia occurred in 3 (9.09%) and 4 (12.12%), vomiting in 23 (70%) and 19 (58%), and blood transfusion in 12

(36.6%) and 13 (39.4%), respectively. One half of a pair of twins was reported in 3 (9.09%) neonates in each group, and consanguineous parentage in 3 (9.09%) suspected NEC and 2 (2.06%) non-NEC neonates. CHD was present in 13 (39.4%) suspected NEC and 11 (33.3%) non-NEC neonates. Sepsis was recorded in 29 (87.88%) suspected NEC and 24 (72.73%) non-NEC neonates. Cardiogenic shock was observed in 1 (3.03%) and 2 (6.06%), and seizures in 3 (9.09%) and 5 (15.15%), respectively. The mean number of antibiotics prescribed was 4.18 in the suspected NEC group and 3.12 in the non-NEC group **(Table 3.2)**.

3.3 Estimation of Bacterial Load

The following tables show the CFU counts from stool culture samples of suspected NEC cases and non-NEC controls. CFU counts were measured on UTI agar, Mannitol Salt Agar (MSA), Blood agar, and Nutrient agar (NA) to observe bacterial growth across selective and non-selective media. For each medium, the average CFU/mL is also included to provide an overall comparison of bacterial load between the two groups (**Table 3.3 and 3.4**).

Table 3.3: Bacterial Load Suspected NEC Samples

Suspected NEC	**UTI**	**MSA**	**Blood Agar**	**NA**
ADNEC-75	$5.7x10^2$	$1x10^2$	$7x10^3$	$8.50x10^2$
ADNEC-77	$4.2x10^4$	$5.2x10^4$	0	$2.10x10^2$
ADNEC-79	$4.6x10^3$	$5.2x10^2$	$1.75x10^4$	$1.34x10^4$
ADNEC-81	$9.90x10^5$	0	$2x10^2$	$7.50x10^5$
ADNEC-83	$2x10^7$	$1x10^3$	0	$4.10x10^6$
ADNEC-85	$2.30x10^6$	$5.7x10^3$	$9.8x10^6$	$1x10^7$
ADNEC-87	$5.8x10^6$	$4.9x10^2$	$8.2x10^6$	$9.90x10^6$
ADNEC-89	$2x10^5$	0	$3.10x10^6$	$2x10^7$
ADNEC-91	$5.90x10^8$	$2.4x10^3$	$9.6x10^6$	$8.40x10^6$
ADNEC-93	$7x10^5$	$2.4x10^2$	$3x10^6$	$3.20x10^6$
ADNEC-95	$1.25x10^7$	$2.4x10^4$	$1.5x10^7$	$4.30x10^6$
ADNEC-97	$1x10^7$	$1x10^2$	$9x10^7$	$2.20x10^7$
ADNEC-99	$3.10x10^6$	$1.72x10^3$	$8x10^5$	$1.30x10^6$
ADNEC-101	$1.80x10^6$	$1.12x10^4$	$1.65x10^7$	$3.60x10^7$
ADNEC-103	$5.50x10^7$	$1.5x10^4$	$7.70x10^7$	$1.51x10^7$
ADNEC-105	$3x10^5$	$5x10^1$	$1.10x10^6$	$1.40x10^5$
ADNEC-107	$9.20x10^6$	$1.2x10^2$	$1.05x10^7$	$1.25x10^6$
ADNEC-109	$8.30x10^6$	$1.01x10^3$	$1.60x10^6$	$1.35x10^5$
ADNEC-111	$1.02x10^7$	$1.4x10^3$	$1.25x10^7$	$2.25x10^6$
ADNEC-113	$4.40x10^6$	$2.01x10^3$	$2.10x10^7$	2.53E+07
ADNEC-115	$1x10^5$	$6x10^1$	$5x10^7$	$3x10^4$
ADNEC-117	$1.90x10^8$	$4.85x10^3$	$1.6x10^8$	$1.02x10^8$
ADNEC-119	$3.80x10^8$	$3x10^1$	$1.7x10^8$	$1.70x10^5$
ADNEC-121	$9x10^4$	$1.6x10^3$	$2.20x10^4$	$1.01x10^6$
ADNEC-123	$4x10^7$	$5.3x10^3$	$5x10^7$	$1.20x10^7$
ADNEC-125	$3x10^6$	$8.2x10^2$	$5.50x10^6$	$2.20x10^6$
ADNEC-127	$7.60x10^8$	$4x10^1$	$4.60x10^8$	$4.60x10^7$
ADNEC-129	$2x10^7$	$2.9x10^2$	$4.7x10^8$	$3.x10^5$
ADNEC-131	$9.70x10^6$	$2.90x10^2$	$1x10^8$	$1.45x10^6$
ADNEC-133	$4.30x10^8$	$1.21x10^4$	$5.70x10^8$	$4.50x10^7$
ADNEC-135	$2x10^6$	$4.7x10^3$	$2.40x10^6$	$1.70x10^5$
ADNEC-137	$6x10^7$	$3x10^1$	$8.50x10^5$	$8.30x10^6$
ADNEC-139	$5x10^8$	$9.5x10^3$	$6.40x10^8$	$2.90x10^7$
Average CFU/mL	$9.5x10^7$	$4.84x10^3$	$8.97x10^7$	$1.25x10^7$

Table 3.4: Bacterial Load of Non-NEC Samples

Non-NEC	**UTI**	**MSA**	**Blood Agar**	**NA**
ADNEC-76	$8x10^{6}$	$3x10^{2}$	0	$2.40x10^{6}$
ADNEC-78	$7.6x10^{8}$	$1x10^{2}$	$2.80x10^{9}$	$5.70x10^{6}$
ADNEC-80	$1.81x10^{7}$	0	$3.30x10^{7}$	$7.60x10^{6}$
ADNEC-82	$2.15x10^{5}$	$1.50x10^{3}$	0	$4.20x10^{5}$
ADNEC-84	$1.70x10^{6}$	$3.89x10^{4}$	$1.42x10^{9}$	$1.10x10^{6}$
ADNEC-86	$1.25x10^{7}$	0	$1.50x10^{9}$	$7.50x10^{6}$
ADNEC-88	$1.40x10^{6}$	$2.16x10^{4}$	$3.30x10^{6}$	$2.70x10^{6}$
ADNEC-90	$9x10^{6}$	$2.30x10^{2}$	$4x10^{5}$	$2.50x10^{6}$
ADNEC-92	$1.10x10^{6}$	$3x10^{1}$	$2.20x10^{6}$	$2.60x10^{7}$
ADNEC-94	$2.20x10^{6}$	$2.10x10^{2}$	$3.80x10^{6}$	$5.40x10^{5}$
ADNEC-96	0	0	$2x10^{7}$	$2x10^{6}$
ADNEC-98	$7.4x10^{6}$	$2x10^{2}$	$1.22x10^{5}$	$2.04x10^{5}$
ADNEC-100	$1.09x10^{7}$	$9.5x10^{2}$	$1.13x10^{6}$	$1.50x10^{7}$
ADNEC-102	$7.50x10^{6}$	$2.21x10^{3}$	$9.70x10^{6}$	$1.02x10^{6}$
ADNEC-104	$6.40x10^{6}$	0	$1.08x10^{7}$	$4x10^{5}$
ADNEC-106	$2.90x10^{8}$	$2.70x10^{3}$	$2.90x10^{8}$	$5.30x10^{7}$
ADNEC-108	$5x10^{3}$	$1.40x10^{2}$	$1.04x10^{4}$	$1.50x10^{2}$
ADNEC-110	$1.05x10^{5}$	$1.22x10^{3}$	$1.30x10^{5}$	$2.04x10^{4}$
ADNEC-112	$2.60x10^{6}$	$6x10^{2}$	$4.20x10^{6}$	$2.10x10^{7}$
ADNEC-114	$2x10^{6}$	$5.70x10^{2}$	$1.10x10^{6}$	$3.40x10^{5}$
ADNEC-116	$7.80x10^{6}$	$8x10^{1}$	$1.11x10^{7}$	$4x10^{5}$
ADNEC-118	$8.90x10^{8}$	$3.80x10^{3}$	$1.18x10^{9}$	$1.20x10^{8}$
ADNEC-120	$1.64x10^{5}$	$1x10^{1}$	$1.22x10^{7}$	$1.20x10^{7}$
ADNEC-122	$6.30x10^{8}$	$6.10x10^{3}$	$4.73x10^{4}$	$1.02x10^{8}$
ADNEC-124	$4.30x10^{8}$	0	$4.20x10^{8}$	$5.80x10^{7}$
ADNEC-126	$1.80x10^{6}$	$9x10^{1}$	$2.90x10^{4}$	$1.09x10^{6}$
ADNEC-128	$1.10x10^{9}$	$2.01x10^{4}$	$2.36x10^{9}$	$1.24x10^{8}$
ADNEC-130	$4.90x10^{6}$	$2.30x10^{3}$	$4.60x10^{6}$	$2x10^{7}$
ADNEC-132	$5.90x10^{8}$	$2x10^{1}$	$2.30x10^{8}$	$1.07x10^{8}$
ADNEC-134	$6.50x10^{6}$	$1x10^{2}$	$2.30x10^{4}$	$1.10x10^{5}$
ADNEC-136	$2.20x10^{8}$	$3x10^{1}$	$2.60x10^{8}$	$1x10^{5}$
ADNEC-138	$3.90x10^{8}$	$5x10^{3}$	$3.60x10^{8}$	$2.80x10^{7}$
ADNEC-140	$7x10^{6}$	$1x10^{2}$	$5.90x10^{7}$	$7.70x10^{6}$
Average CFU/mL	$1.79x10^{7}$	$6.06x10^{1}$	$6.97x10^{6}$	$3.24x10^{6}$

3.4 Presumptive Identification of Bacteria

Presumptive identification of organisms was performed from MSA, UTI, and BAP media based on colony morphology.

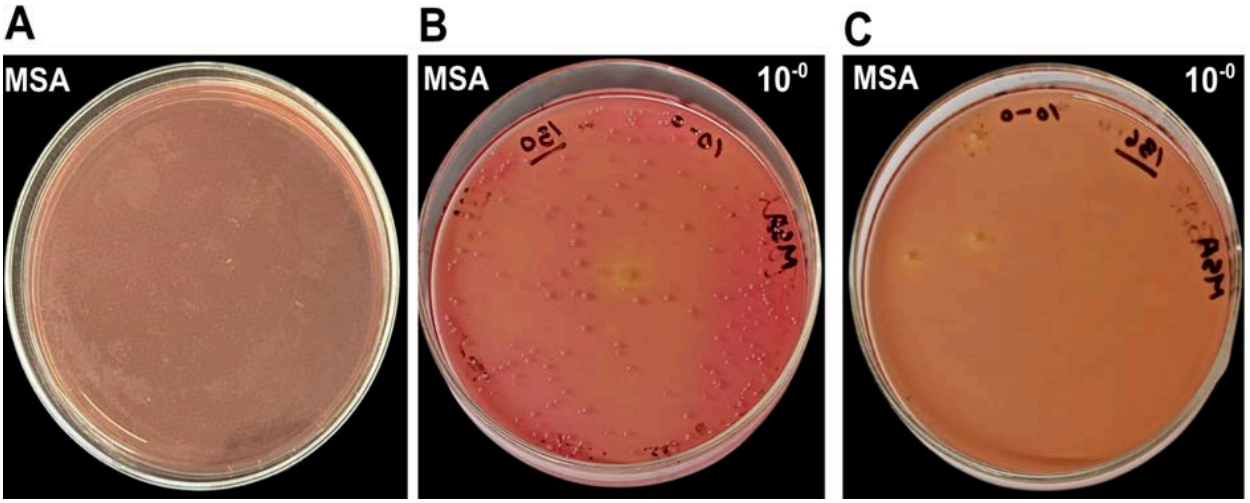

Figure 3.1: (A) MSA media; (B) Mannitol Non- Fermenting *Staphylococcus aureus*; (C) Mannitol Fermenting *Staphylococcus epidermidis*.

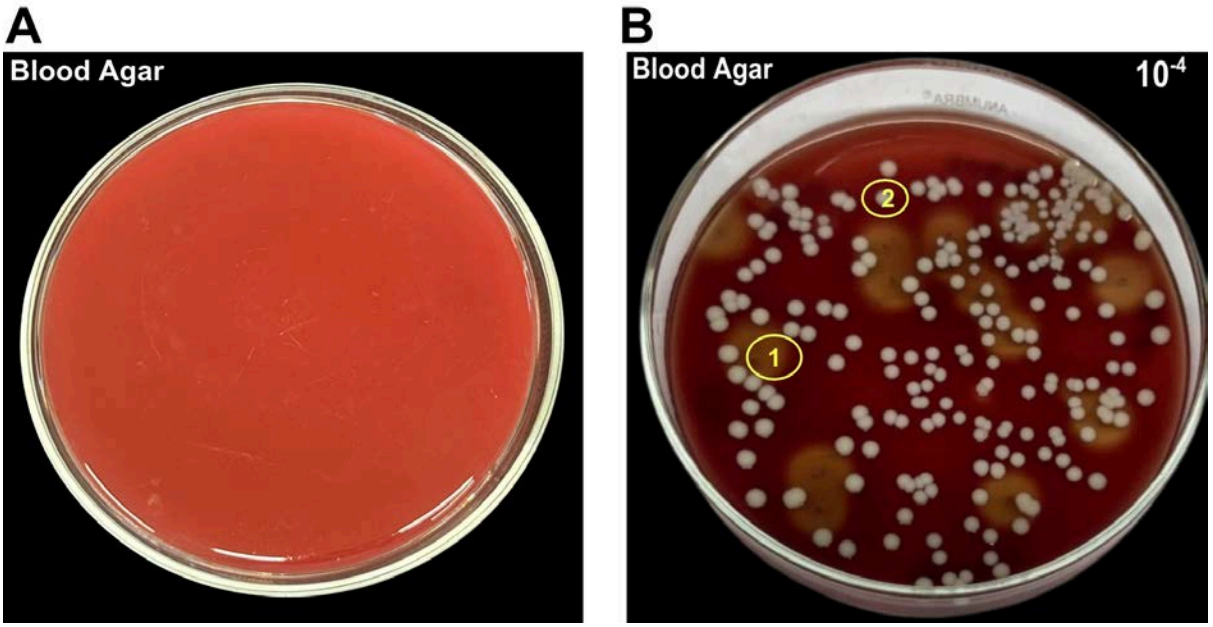

Figure 3.2 : (A) Blood Agar ; (B) Beta Hemolysis (1), *K. pneumoniae* (2).

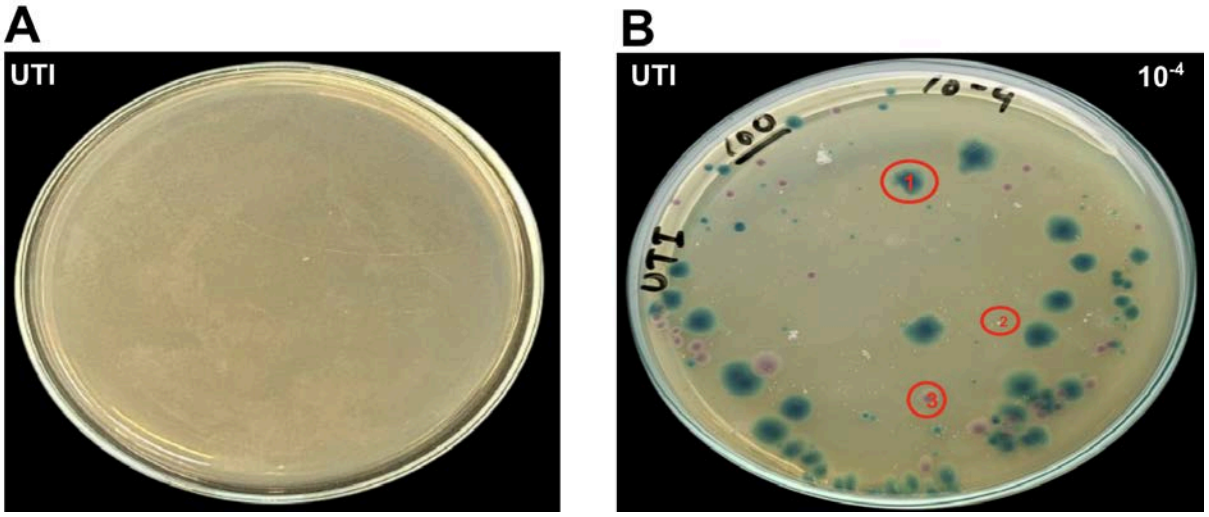

Figure 3.3: (A) UTI Media ; (B) *K. pneumoniae* (1), *S. saprophyticus* (2), *E.coli* (3)

These figures show representative colony growth and presumptive identification on selective and differential media. On MSA, mannitol non-fermenting colonies consistent with *Staphylococcus aureus* and mannitol fermenting colonies consistent with *Staphylococcus saprophyticus* were observed **(Figure 3.1)**. On blood agar, beta-hemolytic growth was demonstrated, and colonies consistent with *Klebsiella pneumoniae* were noted **(Figure 3.2)**. On HiCrome UTI agar, colonies showing characteristic chromogenic appearances consistent with *K. pneumoniae*, *S. saprophyticus*, and *Escherichia coli* were observed **(Figure 3.3)**.

The following table shows the bacterial species identified based on selective media.

Table 3.5: Suspected Bacterial species found in Suspected NEC Cases on Selective Media

Sample Number	***E. coli***	***K. pneumoniae***	***S. Saprophyticus***	***S. aureus***	***S. epidermidis***	***P. aeruginosa***	**Others**
ADNEC-75	-	+	-	+	+	-	+
ADNEC-77	-	+	-	+	+	-	+
ADNEC-79	-	-	-	+	-	+	+
ADNEC-81	-	+	-	+	+	-	+
ADNEC-83	-	+	-	+	+	-	+
ADNEC-85	+	-	-	+	+	+	-
ADNEC-87	+	-	-	+	-	+	-
ADNEC-89	+	+	-	-	-	-	+
ADNEC-91	+	+	-	+	-	-	+
ADNEC-93	+	+	-	+	-	-	+
ADNEC-95	-	+	-	+	-	-	-
ADNEC-97	+	+	-	+	-	-	-
ADNEC-99	+	-	-	+	-	-	-
ADNEC-101	-	+	-	+	-	-	+
ADNEC-103	+	+	+	+	-	-	-
ADNEC-105	-	+	-	+	-	-	-
ADNEC-107	-	+	+	+	-	-	+
ADNEC-109	-	+	+	+	-	+	-
ADNEC-111	-	+	-	+	-	-	+
ADNEC-113	-	+	-	+	-	-	+
ADNEC-115	-	+	-	+	-	-	-
ADNEC-117	-	+	+	+	-	-	-
ADNEC-119	-	-	-	+	-	-	-
ADNEC-121	-	+	+	+	-	-	+
ADNEC-123	-	+	-	+	-	-	-
ADNEC-125	-	+	+	+	-	-	+
ADNEC-127	+	+	-	+	-	-	-
ADNEC-129	-	+	-	+	-	-	-
ADNEC-131	+	+	-	+	-	-	+
ADNEC-133	-	+	+	+	-	-	+
ADNEC-135	-	+	-	+	-	+	+
ADNEC-137	-	+	-	+	-	-	-
ADNEC-139	-	+	-	+	-	-	-
Frequency	10	28	7	32	5	5	17
Percentage	30.3	84.8	21.2	97.0	15.2	15.2	51.5

Table 3.6: Suspected Bacterial species found in Non-NEC Controls on Selective Media

Sample Number	***E. coli***	***K. pneumoniae***	***S. Saprophyticus***	***S. aureus***	***S. epidermidis***	***P. aeruginosa***	**Others**
ADNEC-76	+	+	-	+	+	-	+
ADNEC-78	-	+	-	+	+	+	+
ADNEC-80	-	+	-	+	+	+	+
ADNEC-82	+	+	-	+	-	-	+
ADNEC-84	-	-	-	+	-	+	-
ADNEC-86	-	-	-	+	-	+	-
ADNEC-88	+	+	-	+	-	-	+
ADNEC-90	-	+	-	+	-	-	-
ADNEC-92	-	+	-	+	-	-	-
ADNEC-94	-	+	-	+	-	-	+
ADNEC-96	-	+	-	+	-	-	+
ADNEC-98	-	+	-	+	-	-	-
ADNEC-100	+	+	-	+	-	-	-
ADNEC-102	-	+	-	+	+	-	-
ADNEC-104	-	+	-	-	-	-	+
ADNEC-106	-	+	-	+	-	-	+
ADNEC-108	-	-	-	+	-	+	+
ADNEC-110	-	+	+	+	-	-	-
ADNEC-112	-	+	-	+	-	+	+
ADNEC-114	-	+	-	+	-	-	+
ADNEC-116	-	+	-	+	-	-	-
ADNEC-118	+	+	+	+	-	-	-
ADNEC-120	-	+	-	+	-	-	-
ADNEC-122	-	+	+	+	-	-	-
ADNEC-124	-	+	-	+	-	-	-
ADNEC-126	-	+	-	+	-	+	-
ADNEC-128	-	+	+	+	-	-	-
ADNEC-130	-	+	+	+	-	+	+
ADNEC-132	-	+	-	+	-	+	+
ADNEC-134	-	+	+	+	-	+	+
ADNEC-136	-	+	+	+	-	+	+
ADNEC-138	+	+	-	+	-	-	+
ADNEC-140	+	+	+	+	-	-	+
Frequency	7	30	8	32	4	11	18
Percentage	21.2	90.9	24.2	97.0	12.1	33.3	54.5

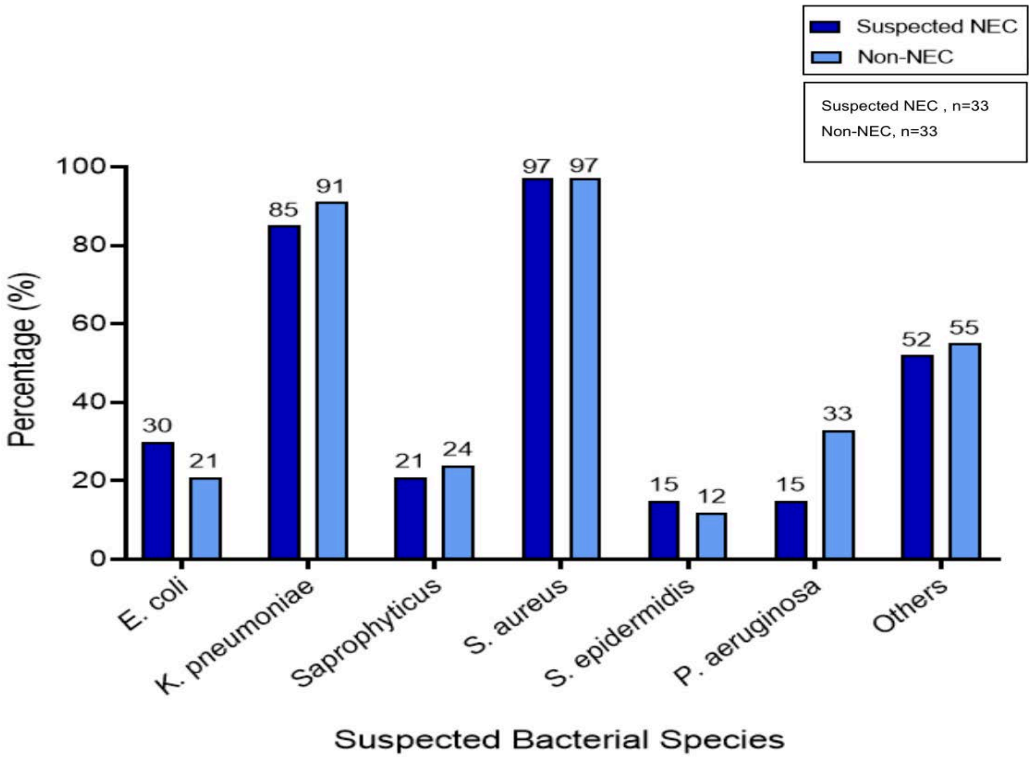

Figure 3.4 : Presumptive Identification of Suspected Bacterial Isolates.

Figure 3.4 presents the presumptive identification of bacterial isolates recovered from suspected NEC and non-NEC groups (n = 33 each). *Staphylococcus aureus* was detected in 97% of samples from both groups. *Klebsiella pneumoniae* was identified in 85% of suspected NEC cases and 91% of non-NEC cases. *Escherichia coli* was observed in 30% of suspected NEC and 21% of non-NEC samples. *Staphylococcus saprophyticus* was detected in 21% and 24% of isolates, respectively. *Staphylococcus epidermidis* was identified in 15% of suspected NEC and 12% of non-NEC samples. *Pseudomonas aeruginosa* was detected in 15% of suspected NEC and 33% of non-NEC samples. Other bacterial species were observed in 52% of suspected NEC and 55% of non-NEC samples.

3.5 PCR and Gel Electrophoresis Results

The PCR and gel electrophoresis confirmation results for isolates from the suspected NEC and non-NEC groups. In UTI medium, *K. pneumoniae* was confirmed in 15 suspected NEC samples and 4 non-NEC samples, while *E. coli* was confirmed in 7 suspected NEC samples and 3 non-NEC samples (**Figure 3.5**). On the other hand, in BAP medium, *K. pneumoniae* was confirmed in 8 suspected NEC samples and 13 non-NEC samples (**Figure 3.6**).

Table 3.7 : PCR and Gel Electrophoresis Results

Confirmed Bacteria	**Suspected NEC**	**Non-NEC**
K. pneumoniae (UTI Medium)	15	4
K. pneumoniae (BAP Medium)	8	13
E.coli	7	3

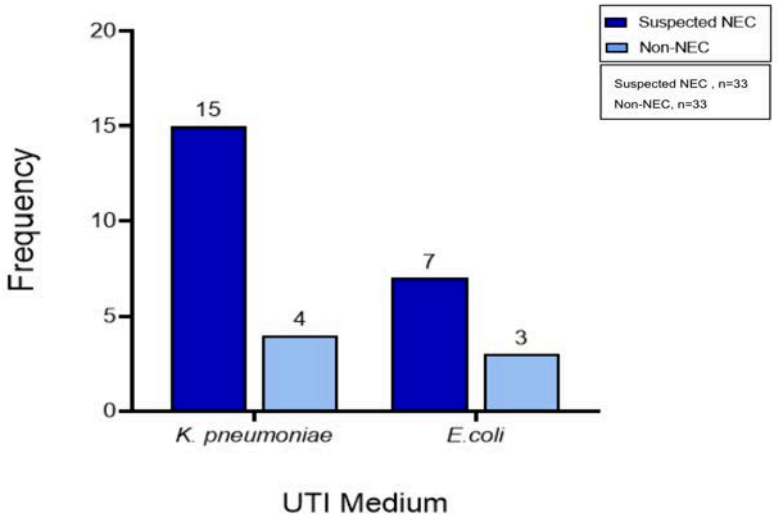

Figure 3.5 : PCR Confirmed *K.pneumoniae* and *E.coli* in UTI Medium.

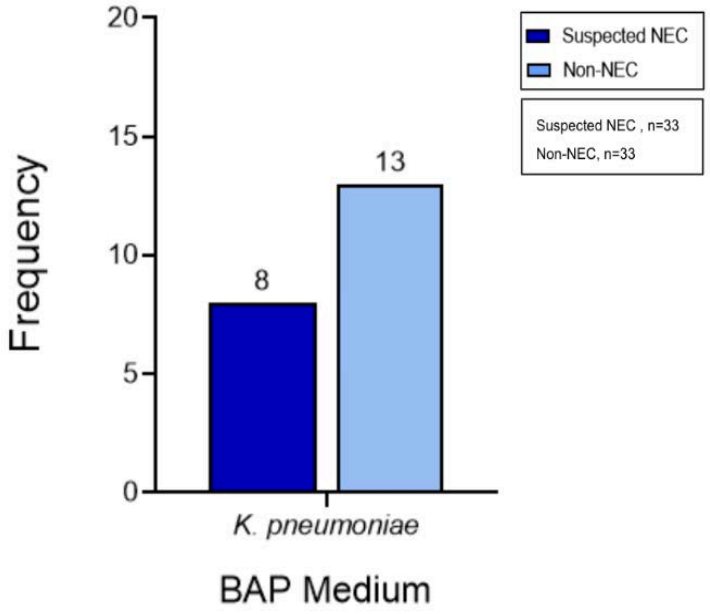

Figure 3.6 : PCR Confirmed *K. pneumoniae* in BAP Medium.

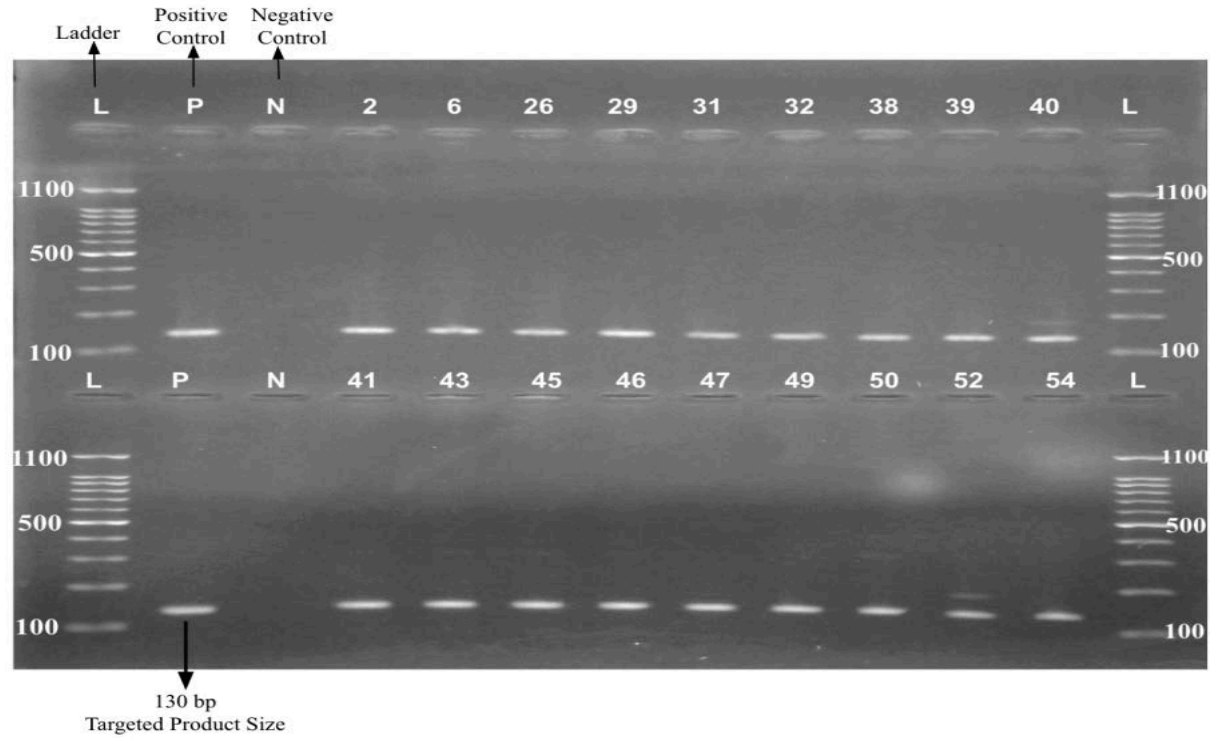

Figure 3.7 : PCR Confirmation of *K. pneumoniae* using Gel Electrophoresis.

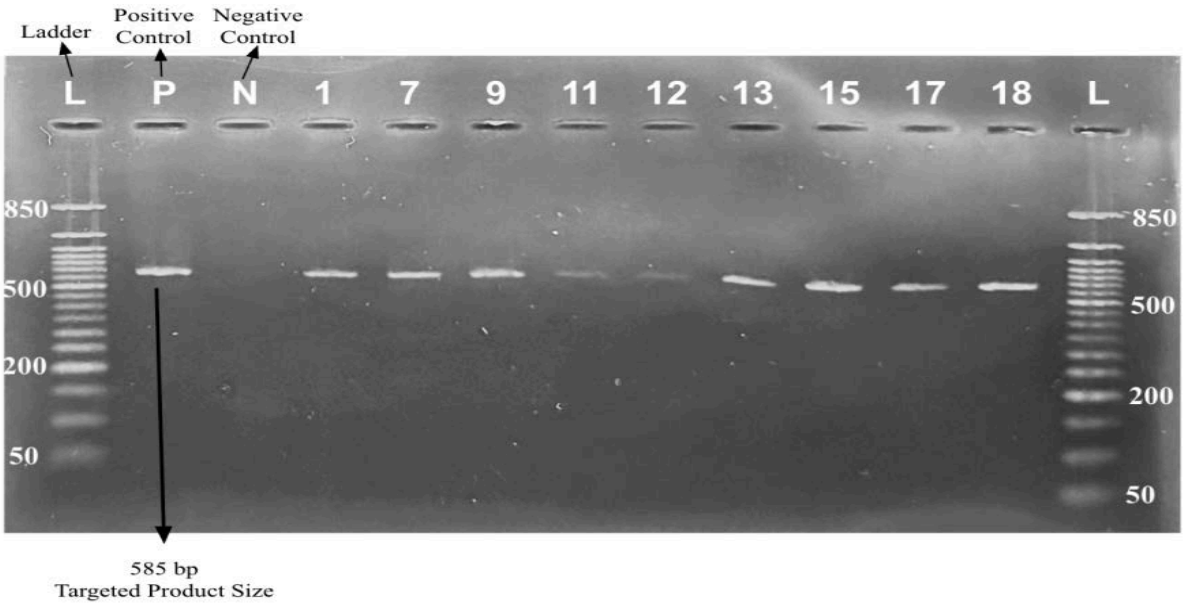

Figure 3.8 : PCR Confirmation of *E. coli* using Gel Electrophoresis.

3.6 CFU/mL Comparison on NA Media Between Suspected NEC and Non-NEC Groups

The total aerobic bacterial load was compared between suspected NEC and non-NEC groups using nutrient agar.

Table 3.8 : CFU/mL Comparison on NA Media Between Suspected NEC and Non-NEC Groups

Characteristics	**Average CFU/mL Count on NA Media**
Suspected NEC	$1.25x10^7$
Non-NEC	$2.21x10^7$
Ratio of NA	1.8 times higher than Suspected NEC

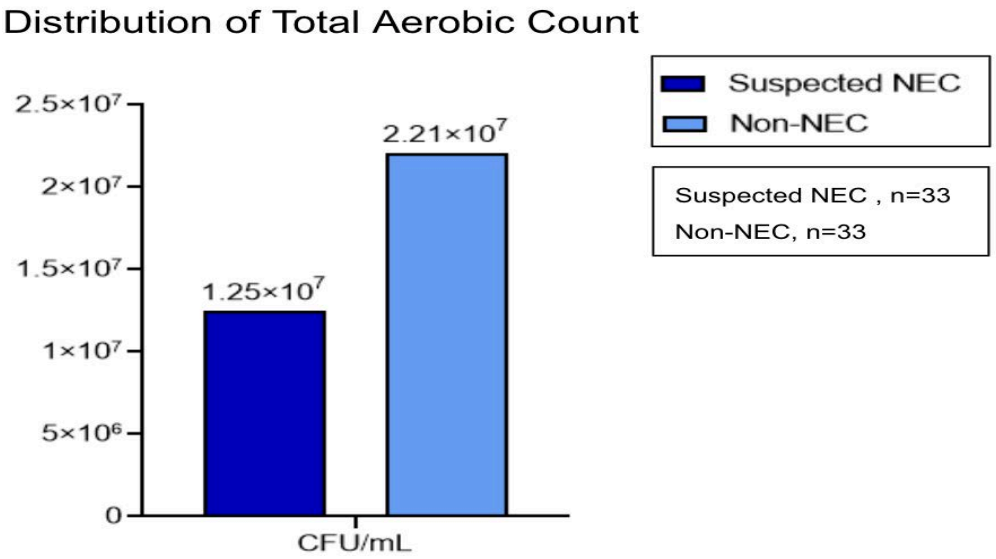

Figure 3.9: Prevalence of Total Aerobic Count.

This shows that non-NEC (control) isolates have higher bacterial growth on nutrient agar than suspected NEC (case) isolates. The average CFU/mL for suspected NEC isolates is approximately 1.25×10^7, while non-NEC isolates reach about 2.21×10^7 CFU/mL. This means bacterial growth in non-NEC isolates is about 1.8 times higher than in suspected NEC isolates.

3.7 Antibiotic Resistance Profile

The antibiotic susceptibility patterns of *Klebsiella pneumoniae* and *Escherichia coli* isolates were analyzed to assess resistance trends in suspected NEC and non-NEC groups.

Table 3.9 : Antibiotic Susceptibility of *K. pneumoniae* in Suspected NEC (UTI Medium, n=15)

Antibiotics	Resistant	Intermediate	Sensitive
K (30)	13 (87%)	1 (7%)	2 (25%)
CFM (5)	15 (100%)	0 (0%)	0 (0%)
ATM (30)	10 (67%)	0 (0%)	5 (33%)
FEP (30)	8 (53%)	2 (13%)	5 (33%)
AMP (10)	14 (93%)	0 (0%)	1 (7%)
IPM (10)	7 (47)%)	2 (13%)	6 (40%)
TE (30)	9 (60%)	0 (0%)	6 (40%)
AMC (30)	15 (100%)	0 (0%)	0 (0%)
CIP (5)	9 (60%)	3 (20%)	3 (20%)

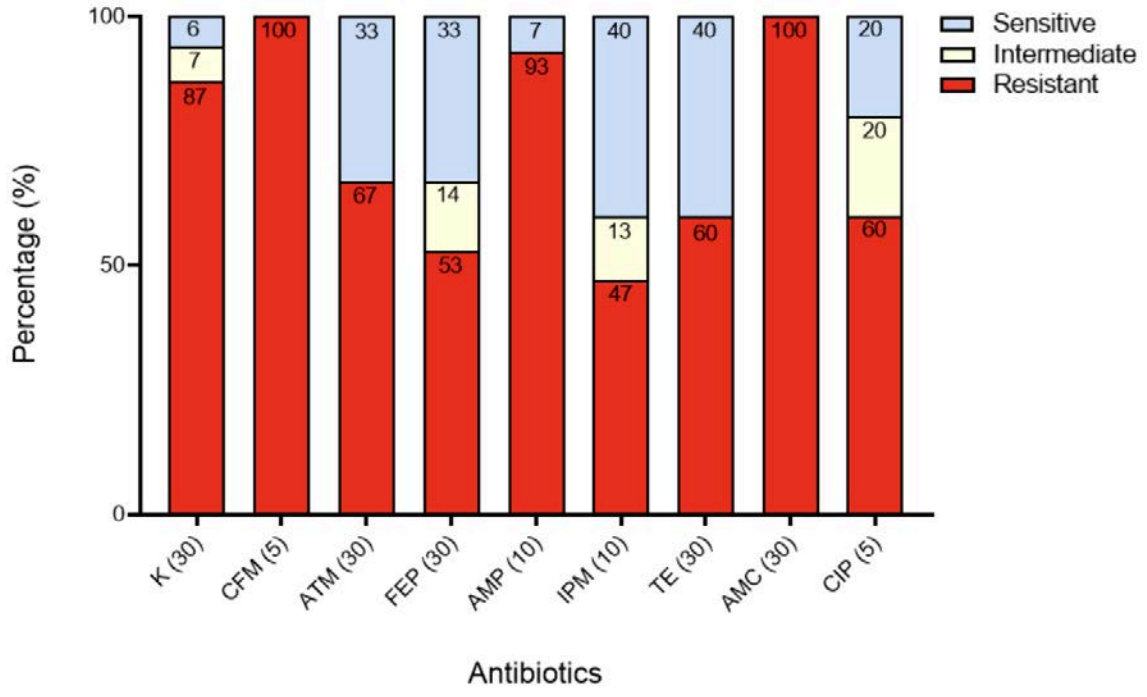

Figure 3.10: Antibiotic Susceptibility of *K. pneumoniae* in Suspected NEC (UTI Medium).

These present the antibiotic susceptibility profile of *Klebsiella pneumoniae* isolates recovered from suspected NEC cases using UTI medium (n = 15). Resistance was observed to K (87%),

CFM (100%), ATM (67%), FEP (53%), AMP (93%), IPM (47%), TE (60%), AMC (100%), and CIP (60%). Intermediate susceptibility was noted for K (7%), FEP (13%), IPM (13%), and CIP (20%). Sensitivity was recorded for K (25%), ATM (33%), FEP (33%), AMP (7%), IPM (40%), TE (40%), and CIP (20%), while no sensitivity was observed for CFM and AMC.

Table 3.10 : Antibiotic Susceptibility of *K. pneumoniae* in Non- NEC (UTI Medium, n=4)

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
K (30)	4 (100%)	0 (0%)	0 (0%)
CFM (5)	4 (100%)	0 (0%)	0 (0%)
ATM (30)	3 (75%)	0 (0%)	1 (25%)
FEP (30)	2 (50%)	0 (0%)	2 (50%)
AMP (10)	4 (100%)	0 (0%)	0 (0%)
IPM (10)	2 (50)%)	0 (0%)	2 (50%)
TE (30)	1 (25%)	0 (0%)	3 (75%)
AMC (30)	4 (100%)	0 (0%)	0 (0%)
CIP (5)	1 (25%)	1(25%)	2 (50%)

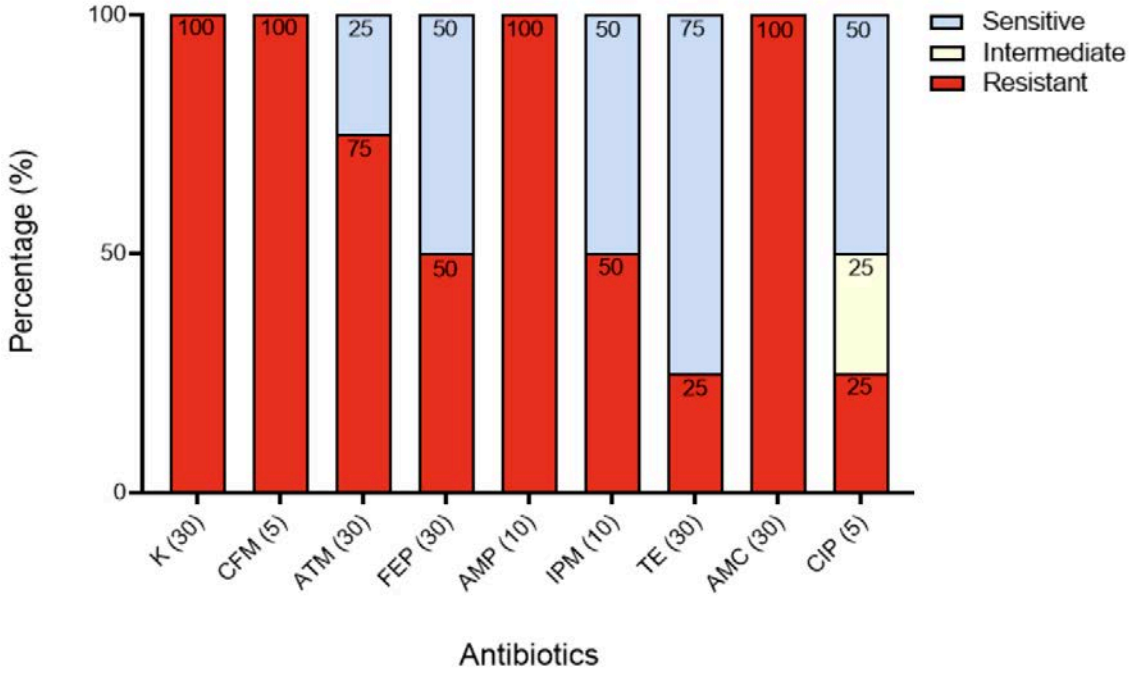

Figure 3.11: Antibiotic Susceptibility of K. *pneumoniae* in Non- NEC (UTI Medium).

These summarize the antibiotic susceptibility profile of *Klebsiella pneumoniae* isolates from the non-NEC group using UTI medium (n = 4). All isolates were resistant to K, CFM, AMP,

and AMC (100% each). Resistance to ATM and FEP was observed in 75% and 50% of isolates, respectively. IPM showed equal proportions of resistance and sensitivity (50% each). TE demonstrated sensitivity in 75% of isolates and resistance in 25%. For CIP, sensitivity was observed in 50% of isolates, intermediate susceptibility in 25%, and resistance in 25%.

Table 3.11 : Antibiotic Susceptibility of *K. pneumoniae* in Suspected NEC (BAP Medium, n=8)

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
K (30)	3 (38%)	3 (38%)	2 (25%)
CFM (5)	8 (100%)	0 (0%)	0 (0%)
ATM (30)	7 (88%)	1 (13%)	0 (0%)
FEP (30)	8 (100%)	0 (0%)	0 (0%)
AMP (10)	8 (100%)	0 (0%)	0 (0%)
IPM (10)	5 (63)%)	2 (25%)	1 (13%)
TE (30)	3 (38%)	0 (0%)	5 (63%)
AMC (30)	8 (100%)	0 (0%)	0 (0%)
CIP (5)	3 (38%)	1 (13%)	4 (50%)

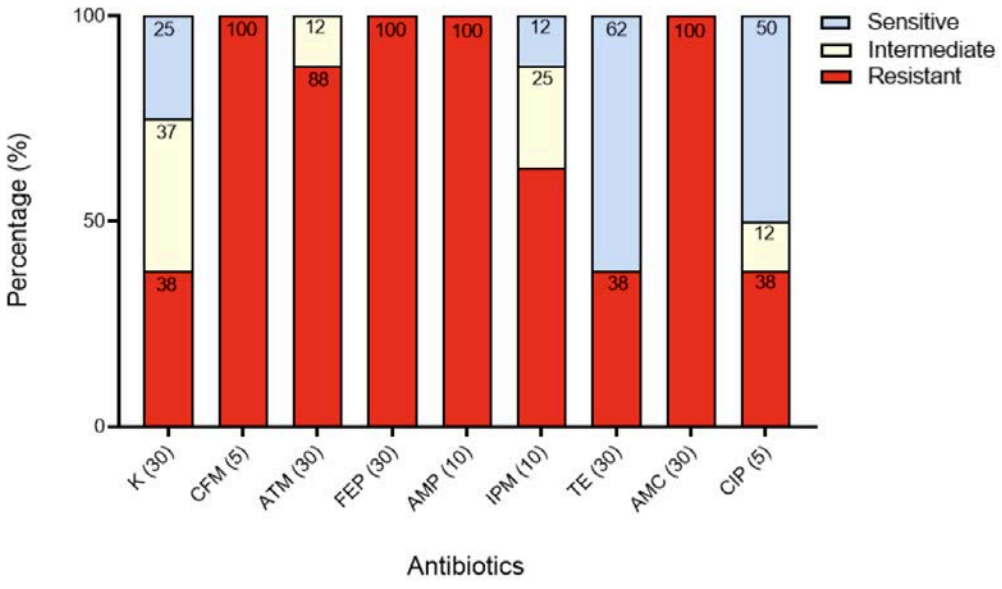

Figure 3.12 : Antibiotic Susceptibility of *K. pneumoniae* in Suspected NEC (BAP Medium).

These present the antibiotic susceptibility profile of *Klebsiella pneumoniae* isolates obtained from suspected NEC cases on BAP medium (n = 8). Resistance was observed for CFM, FEP, AMP, and AMC in all isolates (100%). Resistance to ATM was detected in 7 isolates (88%),

with intermediate susceptibility in 1 isolate (13%). For IPM, resistance, intermediate susceptibility, and sensitivity were observed in 5 (63%), 2 (25%), and 1 (13%) isolates, respectively. For K, resistance and intermediate susceptibility were each observed in 3 isolates (38%), while sensitivity was observed in 2 isolates (25%). TE showed sensitivity in 5 isolates (63%) and resistance in 3 isolates (38%). CIP demonstrated sensitivity in 4 isolates (50%), resistance in 3 isolates (38%), and intermediate susceptibility in 1 isolate (13%).

Table 3.12 : Antibiotic Susceptibility of *K. pneumoniae* in Non- NEC (BAP Medium, n=13)

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
K (30)	4 (100%)	0 (0%)	0 (0%)
CFM (5)	4 (100%)	0 (0%)	0 (0%)
ATM (30)	3 (75%)	0 (0%)	1 (25%)
FEP (30)	2 (50%)	0 (0%)	2 (50%)
AMP (10)	4 (100%)	0 (0%)	0 (0%)
IPM (10)	2 (50)%)	0 (0%)	2 (50%)
TE (30)	1 (25%)	0 (0%)	3 (75%)
AMC (30)	4 (100%)	0 (0%)	0 (0%)
CIP (5)	1 (33%)	0 (0%)	2 (67%)

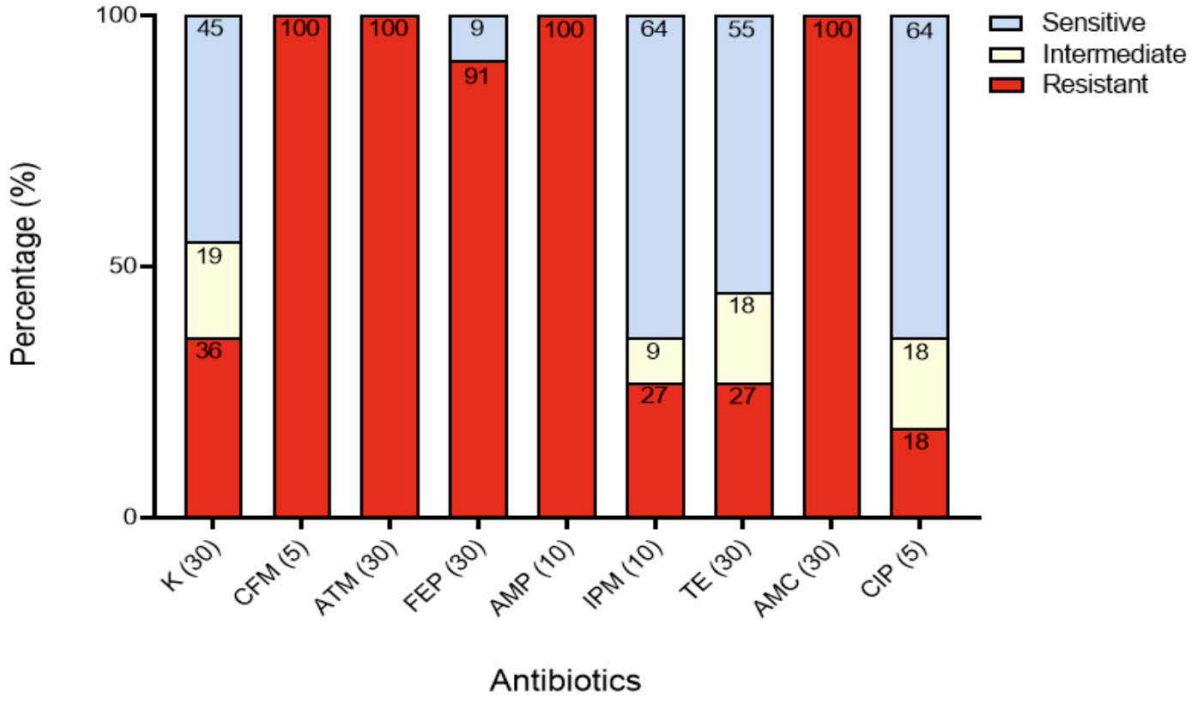

Figure 3.13: Antibiotic Susceptibility of *K. pneumoniae* in Non-NEC (BAP Medium).

Table 3.12 and **Figure 3.13** show the antibiotic susceptibility profile of *Klebsiella pneumoniae* isolates from the non-NEC group cultured on BAP medium (n = 13). All isolates exhibited resistance to K, CFM, AMP, and AMC (100%). Resistance to ATM was observed in 75% of isolates. FEP and IPM showed equal proportions of resistance and sensitivity (50% each). TE demonstrated sensitivity in 75% of isolates and resistance in 25%. CIP showed sensitivity in 67% of isolates and resistance in 33%.

Table 3.13 : Antibiotic Susceptibility of *E.coli* in Suspected NEC (UTI Medium, n=7)

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
K (30)	6 (86%)	0 (0%)	1 (14%)
CFM (5)	7 (100%)	0 (0%)	0 (0%)
ATM (30)	7 (100%)	0 (0%)	0 (0%)
FEP (30)	7 (100%)	0 (0%)	0 (0%)
AMP (10)	7 (100%)	0 (0%)	0 (0%)
IPM (10)	5 (71%)	0 (0%)	2 (29%)
TE (30)	3 (43%)	0 (0%)	4 (57%)
AMC (30)	7 (100%)	0 (0%)	0 (0%)
CIP (5)	6 (86%)	1 (14%)	0 (0%)

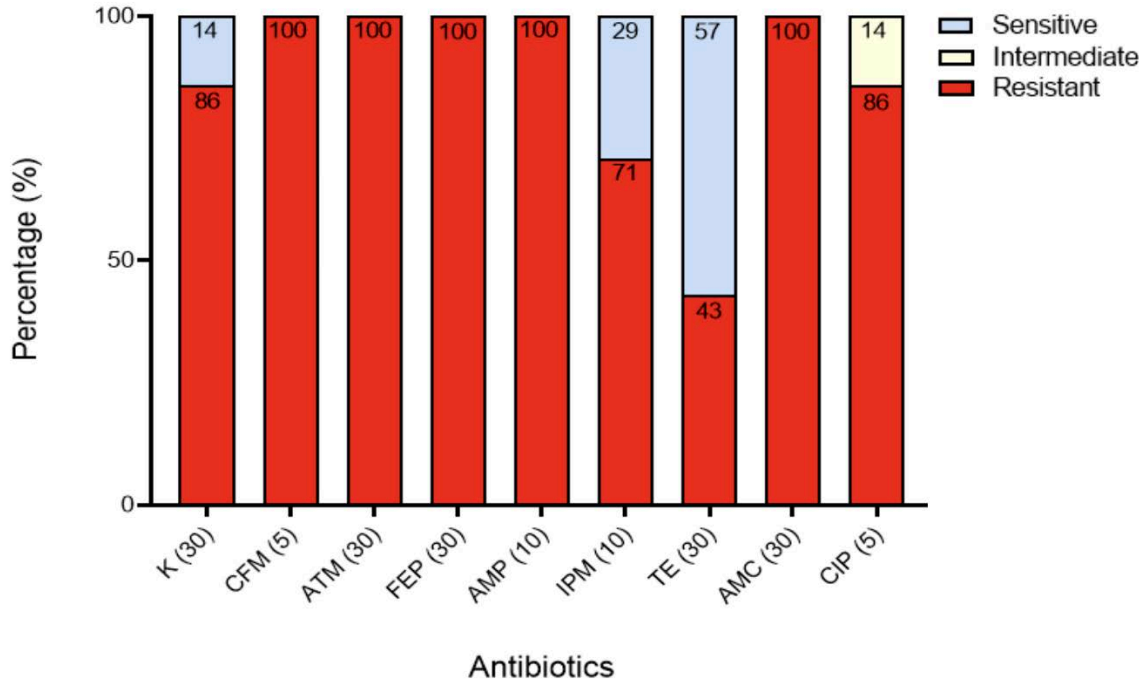

Figure 3.14: Antibiotic Susceptibility of *E.coli* in Suspected NEC (UTI Medium).

These present the antibiotic susceptibility profile of *Escherichia coli* isolates recovered from suspected NEC cases using UTI medium (n = 7). Resistance was observed to CFM, ATM, FEP, AMP, and AMC in all isolates (100%). High resistance was also recorded for K and CIP (86% each). IPM showed resistance in 71% of isolates and sensitivity in 29%. TE demonstrated sensitivity in 57% of isolates and resistance in 43%. Intermediate susceptibility was observed only for CIP in 1 isolate (14%).

Table 3.14 : Antibiotic Susceptibility of E.coli in Non- NEC (UTI Medium, n=3)

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
K (30)	2 (67%)	0 (0%)	1 (33%)
CFM (5)	2 (67%)	0 (0%)	1 (33%)
ATM (30)	2 (67%)	0 (0%)	1 (33%)
FEP (30)	3 (100%)	0 (0%)	0 (0%)
AMP (10)	3 (100%)	0 (0%)	0 (0%)
IPM (10)	2 (67%)	0 (0%)	1 (33%)
TE (30)	1 (33%)	0 (0%)	2 (67%)
AMC (30)	3 (100%)	0 (0%)	0 (0%)
CIP (5)	1 (33%)	0 (0%)	2 (67%)

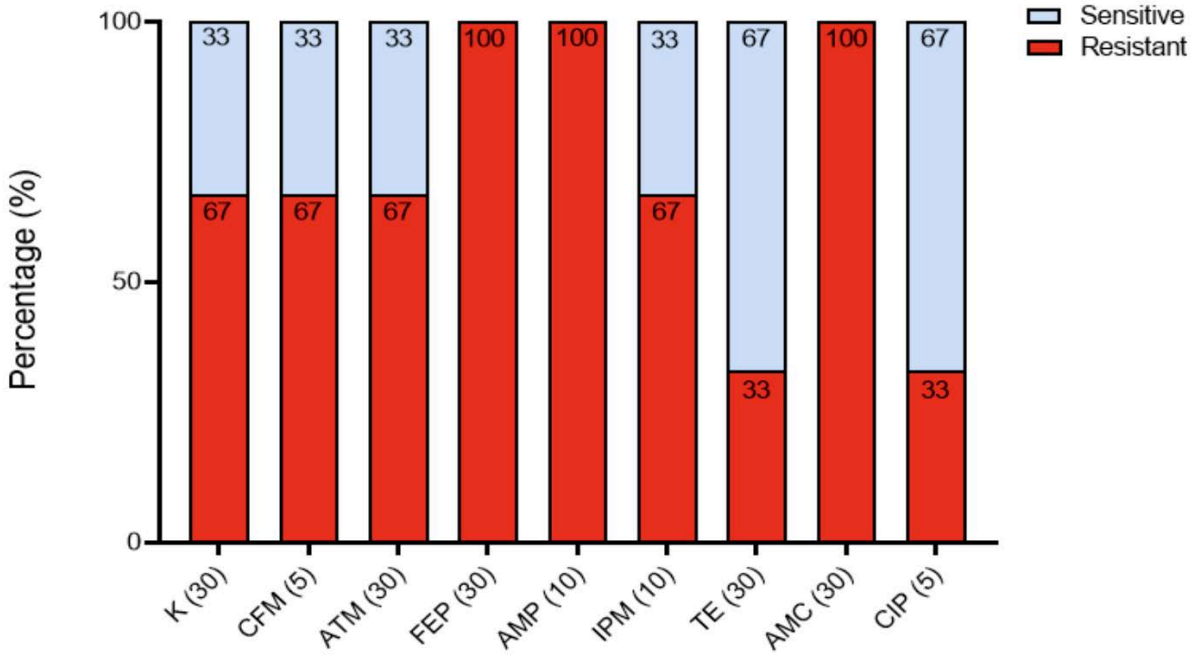

Figure 3.15: Antibiotic Susceptibility of *E.coli* in Non- NEC (UTI Medium).

These show the antibiotic susceptibility profile of *Escherichia coli* isolates from the non-NEC group using UTI medium (n = 3). Resistance was observed to FEP, AMP, and AMC in all

isolates (100%). Resistance to K, CFM, ATM, and IPM was recorded in 67% of isolates, while sensitivity to these antibiotics was observed in 33%. TE demonstrated sensitivity in 67% of isolates and resistance in 33%. CIP showed sensitivity in 67% of isolates and resistance in 33%.

Chapter 4 Discussion

This study explored differences in clinical features, associated risk factors, and gut bacterial characteristics between neonates with suspected NEC and non-NEC controls in Bangladesh. Suspected NEC was identified based on a combination of a positive occult blood test, abdominal distension, and radiological evidence of bubble gas suggestive of pneumatosis intestinalis, which is a well recognized feature of NEC. The aim of the study was to determine how suspected NEC neonates differ from non-NEC controls in terms of clinical presentation, exposure to established risk factors, and gut microbial characteristics. By assessing feeding intolerance, abdominal changes, prematurity, birth weight, antibiotic exposure, and probiotic use alongside stool culture findings and bacterial load estimation, the study sought to identify patterns that may contribute to NEC development.

4.1 Demographic Characteristics

The demographic characteristics of the study population were similar between the suspected NEC and non-NEC groups **(Table 3.1)**. Each group included 33 neonates, indicating appropriate matching and minimal demographic bias. Gender distribution was identical in both groups, with males comprising 51.5% and females 48.5%. This balanced distribution suggests that gender did not influence the comparison between the groups, and the slight male predominance is consistent with previous neonatal findings. Average birth weight was comparable in the suspected NEC and non-NEC groups, measuring 1.28 kg and 1.26 kg respectively, indicating that low birth weight was common in both groups. Gestational age was also similar, with mean values of 33.3 weeks in the suspected NEC group and 33.2 weeks in the non-NEC group, confirming that both groups mainly consisted of preterm neonates.

Overall, the similarity in demographic variables supports the reliability of further analyses, suggesting that any observed differences are more likely related to disease status rather than demographic factors.

4.2 Clinical Characteristics and Associated Risk Factors

Clinical findings and background risk factors were more prominent among suspected NEC infants, supporting the view that NEC develops through a combination of clinical vulnerability and environmental exposures. Feeding difficulty was more frequent among suspected NEC infants, and feeding intolerance is a well recognized early manifestation of

NEC that is often associated with reduced intestinal motility and mucosal injury. Vomiting was also more commonly observed in suspected cases, reflecting impaired gastrointestinal function and delayed gastric emptying during intestinal inflammation.

Abdominal changes were present in all suspected NEC infants and were absent in non-NEC controls. This marked difference highlights abdominal distension and related gastrointestinal abnormalities as key clinical indicators of NEC. These changes are likely a consequence of intestinal inflammation, gas accumulation, and disruption of the gut barrier.

Feeding practices showed that mixed feeding was the most common method in both groups. Exclusive breastfeeding was slightly more frequent among suspected NEC infants, while exclusive formula feeding was more common among non-NEC infants. Although breast milk is known to provide immunological protection and support healthy gut microbiota, these findings indicate that feeding type alone may not be sufficient to prevent NEC when strong predisposing factors such as prematurity and systemic illness are present.

Antibiotic exposure was notably higher among suspected NEC infants, both in terms of the number of prescriptions and the range of antibiotics used. This likely reflects greater disease severity and increased concern for sepsis. At the same time, repeated antibiotic exposure can disrupt normal gut microbial balance by suppressing beneficial commensal bacteria and allowing opportunistic pathogens to dominate. Such antibiotic associated dysbiosis is widely recognized as an important contributor to NEC development.

Low birth weight, very low birth weight, and prematurity were slightly more common among suspected NEC infants. These factors remain central to NEC risk, as intestinal immaturity, reduced immune defense, and weakened mucosal barrier function increase susceptibility to dysbiosis and inflammation. Congenital heart disease and sepsis were also frequently observed and may contribute through impaired gut perfusion, systemic inflammation, and increased vulnerability to infection.

Vitamin D deficiency was more common among non-NEC infants than among suspected NEC cases, suggesting that vitamin D status alone was not a determining factor for NEC development in this cohort. Pneumonia showed no substantial difference between the two groups, indicating a limited association with NEC in this population.

4.3 Presumptive Identification of Bacterial Isolates

Presumptive identification using selective and differential media gave a quick picture of the types of organisms present in the samples. Mannitol Salt Agar, blood agar, and chromogenic

UTI agar were useful because they allow organisms to be differentiated based on visible colony features such as color changes, mannitol fermentation, and hemolysis.

On Mannitol Salt Agar, both mannitol fermenting and non fermenting colonies were seen, suggesting the presence of more than one staphylococcal species. Colonies consistent with *Staphylococcus aureus* and *Staphylococcus saprophyticus* were noted, which is not unexpected in a neonatal environment where skin associated organisms can easily colonize.

On blood agar, beta hemolysis was observed along with colonies consistent with *Klebsiella pneumoniae*. Hemolytic activity is often considered an important indicator in routine microbiology because it reflects how bacteria interact with host tissues and can point toward potentially more pathogenic behavior.

Chromogenic UTI agar further helped in differentiating organisms through characteristic colony colors, with colonies consistent with *K. pneumoniae*, *S. saprophyticus*, and *Escherichia coli* identified. The detection of Gram negative organisms such as *K. pneumoniae* and *E. coli* is clinically relevant because these bacteria are frequently implicated in neonatal infections and have been associated with intestinal inflammation.

Overall, the growth patterns across these media suggest a mixed bacterial population containing both Gram positive and Gram negative organisms. The presence of opportunistic pathogens supports the idea that changes in gut microbial composition may play a role in suspected NEC.

4.4 Total Aerobic Count

Comparison of total aerobic bacterial load using nutrient agar revealed significantly higher CFU per milliliter values in non-NEC samples compared with suspected NEC samples, with counts approximately 1.8 times greater. This indicates a reduced total cultivable aerobic bacterial load among suspected NEC infants.

A lower bacterial count does not necessarily indicate a healthier gut environment. In preterm neonates, normal intestinal development depends on gradual colonization by a diverse range of commensal microorganisms. The reduced bacterial load observed in suspected NEC cases may reflect antibiotic related suppression of normal flora, delayed colonization due to prematurity, or an unstable gut microbial ecosystem. These findings support the view that NEC is more closely associated with microbial imbalance than with absolute bacterial overgrowth.

4.5 PCR Results

PCR analysis was carried out to molecularly confirm bacterial isolates that were initially identified using culture based methods. The presence of *Klebsiella pneumoniae* and *Escherichia coli* was confirmed in both suspected NEC and non-NEC groups, with a higher frequency observed among suspected NEC cases. This finding is clinically important, as both organisms are well known opportunistic pathogens frequently associated with neonatal infections and intestinal inflammation. The PCR results strengthen the culture findings and support the role of pathogenic microbial composition in suspected NEC.

4.6 Antibiotic Susceptibility Patterns in *K. pneumoniae* and *E. coli*

Antibiotic susceptibility testing demonstrated high levels of antimicrobial resistance among *K. pneumoniae* and *E. coli* isolates from both suspected NEC and non-NEC groups. Resistance patterns were generally more severe among isolates from suspected NEC cases, suggesting greater antibiotic pressure or exposure to resistant organisms in the neonatal care environment.

In suspected NEC cases, *K. pneumoniae* showed very high resistance to several commonly used antibiotics, which limits the reliability of standard empiric treatment regimens. In the non-NEC group, resistance was also substantial, indicating that resistant strains may be widely circulating within the neonatal setting. A particularly important concern was reduced susceptibility to imipenem among suspected NEC isolates, given its role as a critical last line therapeutic option.

For *E. coli*, resistance among suspected NEC isolates was extremely high across multiple antibiotics, while non-NEC isolates showed more variable susceptibility with partial sensitivity to several agents. This pattern supports the possibility that suspected NEC is more closely associated with highly resistant strains, which may contribute to persistent colonization and ongoing intestinal inflammation.

Overall, these findings highlight the importance of local antimicrobial resistance surveillance, culture guided therapy when feasible, careful antibiotic stewardship to reduce unnecessary exposure, and effective infection prevention measures to limit the spread of resistant organisms in neonatal units.

4.7 Comparative Synthesis of Our Findings With Published Literature on NEC

These findings are consistent with what has been reported in the NEC literature, where early presentation commonly includes feeding intolerance, vomiting, abdominal distension, and

bloody stool, and bubble gas suggestive of pneumatosis intestinalis is widely regarded as a characteristic diagnostic feature (Thompson & Bizzarro, 2008). The higher detection of *K. pneumoniae* and *E. coli* among suspected NEC cases also matches many reports describing NEC as a condition linked to intestinal dysbiosis, often showing an increase in Enterobacteriaceae or Proteobacteria around the time of disease onset rather than a single fixed causative organism (Fundora et al., 2019). One notable difference in this cohort is the lower total cultivable aerobic bacterial load in suspected NEC compared with non-NEC; however, this can still fit within the dysbiosis model because NEC is frequently described as an imbalance in microbial composition and stability, and antibiotic pressure may suppress overall cultivable flora while allowing specific opportunistic pathogens to dominate. (Fundora et al., 2019b) The higher and broader antibiotic exposure among suspected NEC cases is therefore clinically important and potentially concerning, as several studies have linked prolonged or intensive empiric antibiotic use in very preterm infants with microbiome disruption and increased NEC risk, although illness severity and indication can influence this relationship (Zhu et al., 2022). In contrast, many meta analyses report a protective effect of specific probiotic regimens against NEC, so the lack of a clear protective pattern here may reflect differences in probiotic strains, timing of initiation, dosing, and real world implementation (Han et al., 2025) Finally, the high level of antimicrobial resistance observed is particularly concerning in neonatal care because NEC management often overlaps with sepsis coverage, and resistant gram negative organisms can limit effective empiric treatment and highlight the need for culture guided therapy and antibiotic stewardship whenever possible (Baltogianni et al., 2023).

4.8 Conclusion

In conclusion, suspected NEC in this study was associated with characteristic clinical features and vulnerability related to prematurity and low birth weight, and altered gut microbial dynamics. The reduced total bacterial load observed on nutrient agar supports the role of microbial imbalance rather than bacterial overgrowth in NEC pathogenesis. These findings provide valuable evidence from Bangladesh and emphasize the importance of integrated clinical and microbiological assessment for early recognition and risk evaluation of NEC in high risk neonates.

Chapter 5 References

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Appendix 1

Questionnaire

Project Title: Exploring Necrotizing Enterocolitis (NEC) in Bangladesh.

Participant ID:

Demographic Information of the Participant:

01.	Name of Participant:		
02	Age:		
03.	Gender:	Male	Female
04.	Name of the Parent:		
05.	Occupation of the Parent		
06.	Educational Qualification of the Parent	No education/Less than SSC / SSC or equivalent / HSC or equivalent / Bachelor or equivalent / Masters or equivalent / More than Masters.	
07.	Monthly Income of the Family (BDT):		
08.	Monthly Expenditure of the Family (BDT):		

Clinical Information of the Participant:

01.	Weight (kg):			
02.	Height (cm):			
03.	Types of feeding	Breast feeding		Formula feeding
04.	Has the baby experienced feeding difficulties in recent times?	Yes	No	Please specify:
05.	Did you notice any changes in the baby's abdominal area, such as bloating or distention?	Yes	No	Please specify:
06.	Did you observe any unusual or concerning appearances in the baby's bowel movements? (e.g., presence of gross blood in the stool, gas-filled loop of the bowel)	Yes	No	If yes, how often?
07.	Has the baby had any instances of fever?	Yes	No	If yes, how often?
08.	Has the baby experienced any episodes of vomiting?	Yes	No	If yes, how often?
09.	Has the baby received a blood transfusion after birth?	Yes	No	If yes, how many times?
10.	Has the baby been administered with any intravenous antibiotics after birth?	Yes	No	If yes, name of the antibiotics?
11.	Has the baby been given any probiotics, prebiotics, or both after birth?	Yes	No	Please specify:
12.	Has the baby been given any additional growth factor (e.g., anti-cytokine or glucocorticoids)?	Yes	No	Please specify:

13.	Has the baby been given any intravenous hyperalimentation (A form of nutrition that is delivered into a vein)?	Yes	No	Please specify:
14.	Has the baby undergone any surgical treatment?	Yes	No	If yes, when? Any other information:

History of pregnancy:

01.	Types of delivery	Vaginal birth	C-section	VBAC
02.	Have you experienced any complications during delivery?	Yes	No	If yes, please describe:
03.	Have you experienced any complications during breastfeeding?	Yes	No	If yes, please describe:
04.	Have you experienced any mentionable complications during pregnancy?	Yes	No	If yes, please describe:
05.	Have you been exposed to any particular antibiotic during the times of pregnancy?	Yes	No	If yes, Name of the antibiotics:
06.	Have you used any other drugs during pregnancy or after delivery?	Yes	No	If yes, Name of the antibiotics:

Any other information that you would like to mention:

Participant Information Consent Form

Project Title: Exploring Necrotizing Enterocolitis (NEC) in Bangladesh.

Necrotizing Enterocolitis (NEC) is a devastating fatal disease of preterm infants. This study aims to assess the status of necrotizing enterocolitis (NEC) cases in Bangladesh. In this regard, we need your help. If you agree to participate, please sign below. Thank you.

How will participants be involved in the project?
Parents of the participants will need to provide some information regarding their baby's demography, clinical characteristics and history of the mother's pregnancy. Participants will also provide their stool and blood sample (minimum volume required) for experimental analysis. Blood will be collected by the nurse of the respective hospital.

What are the possible benefits of taking part?
There will be no benefit to participants from participation in this research project. However, it may provide valuable information on the status of NEC in preterm neonates in Bangladesh.

What are the possible risks and disadvantages of taking part?
There will be no risks or harms to participants for taking part in this study. During collection of blood, slight red and swelling around the area of the syringe may happen.

For further information:
Please contact Dr. Nadia Deen (BRAC University) for any query or complaints. Contact number: 01788019434, Email address: nadia.sultana@bracu.ac.bd

Declaration by participant

- √ I have read the Participant Information Consent Form or someone has read it to me in a language that I understand.
- √ I understand the purposes, procedures and risks of this research project.
- √ I agree to donate my baby's stool and blood samples.
- √ I understand that I will be given a signed copy of this document to keep.
- √ I understand that all of my information will remain confidential.

Name of the parent of the participant: Contact number:

Signature of the parent of the participant: Date:

Declaration by researcher
I have given a verbal explanation of the research project, its procedures and risks, and I believe that the parent of the participant has understood that explanation.

Name of the researcher: Signature of the researcher:

সম্মতিপত্র

প্রকল্পের শিরোনাম: বাংলাদেশে নেক্রোটাইজিং এন্টারোকোলাইটিস **(নেক)-**এর অন্বেষণ

নেক্রোটাইজিং এন্টারোকোলাইটিস একটি প্রাণঘাতী রোগ, যা অকাল শিশুদের ক্ষেত্রে দেখা যায়। এই প্রকল্পটিতে বাংলাদেশে নেক্রোটাইজিং এন্টারোকোলাইটিস রোগ সম্পর্কে গবেষণা হবে। এই জন্য আমাদের আপনার সাহায্যের প্রয়োজন। আপনি এই প্রকল্পে আপনার শিশুর অংশগ্রহণে সম্মত হলে, নিচের লেখাগুলি পড়ুন এবং আপনার সই দিন। ধন্যবাদ।

কিভাবে আপনি এবং আপনার শিশু এই প্রকল্পে অংশগ্রহণ করবেন?
অংশগ্রহণকারী শিশুর পিতামাতাকে তাদের শিশুর জনসংখ্যা সংক্রান্ত, ক্লিনিকাল বৈশিষ্ট্য এবং মায়ের গর্ভাবস্থার ইতিহাস সম্পর্কিত কিছু তথ্য প্রদান করতে হবে। পরীক্ষামূলক বিশ্লেষণের জন্য অংশগ্রহণকারীরা তাদের মল এবং রক্তের নমুনা (ন্যূনতম পরিমাণ প্রয়োজন) প্রদান করবে। রক্ত সংগ্রহ করবেন সংশ্লিষ্ট হাসপাতালের নার্স।

এই প্রকল্পে অংশগ্রহণ করলে কি লাভ হবে?
এই গবেষণা প্রকল্পে অংশগ্রহণ করলে অংশগ্রহণকারীদের সরাসরি কোন সুবিধা হবে না। তবে এই প্রকল্পটিতে অংশগ্রহণের মাধ্যমে আপনি এবং আপনার শিশু বাংলাদেশে নবজাতক শিশুদের নেক্রোটাইজিং এন্টারোকোলাইটিস সম্পর্কে মূল্যবান তথ্য প্রদানে সহায়তা করবেন, যা ভবিষ্যতে এই রোগ মোকাবেলায় ভূমিকা রাখবে।

এই গবেষণায় অংশ নেওয়ার সম্ভাব্য ঝুঁকি এবং অসুবিধাগুলি কী কী?
এই গবেষণায় অংশ নেওয়ার জন্য অংশগ্রহণকারীদের কোন ঝুঁকি বা ক্ষতি হবে না। রক্ত সংগ্রহের সময়, সিরিঞ্জের চারপাশে সামান্য লাল এবং ফোলাভাব ঘটতে পারে।

যোগাযোগ:
যেকোন প্রশ্ন বা অভিযোগের জন্য অনুগ্রহ করে ড: নাদিয়া দীনের (ব্র্যাক ইউনিভার্সিটি) সাথে যোগাযোগ করুন। যোগাযোগের নম্বর: ০১৭৮৮০১৯৪৩৪, ইমেল ঠিকানা: nadia.sultana@bracu.ac.bd

অংশগ্রহণকারী ঘোষণা করছেন যে-
√ আমি এই সম্মতিপত্রটি পড়েছি।
√ আমি এই গবেষণা প্রকল্পের উদ্দেশ্য, পদ্ধতি এবং ঝুঁকি সম্পর্কে অবহিত।
√ আমি এই গবেষণা প্রকল্প সম্পর্কে প্রশ্ন করার সুযোগ পেয়েছি এবং যে উত্তর পেয়েছি তাতে আমি সন্তুষ্ট।
√ আমি বুঝতে পারছি যে আমাকে এই নথির একটি স্বাক্ষরিত কপি দেওয়া হবে।
√ আমি বুঝি যে আমার সমস্ত তথ্য গোপন থাকবে।

অংশগ্রহণকারীর নাম: অংশগ্রহণকারীর স্বাক্ষর: তারিখ:

গবেষক ঘোষণা করছেন যে-
আমি উপরোক্ত অংশগ্রহণকারীকে এই গবেষণা প্রকল্প, এর পদ্ধতি এবং ঝুঁকির একটি মৌখিক ব্যাখ্যা দিয়েছি এবং আমি বিশ্বাস করি যে অংশগ্রহণকারী সেই ব্যাখ্যাটি বুঝতে পেরেছেন।

গবেষকের নাম: গবেষকের স্বাক্ষর: তারিখ:

Appendix 2

Table 1: AST Results of *K. pneumoniae* in Suspected NEC (BAP Medium, n=8)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	11	10	10	10	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	21	21	21	21	Intermediate
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-131	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	9	9	9	9	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	31	30	30	30	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	10	10	10	10	Resistant
ADNEC-107	CIP (5)	0	0	0	0	Resistant
	K (30)	22	22	22	22	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	32	27	27	29	Sensitive
	TE (30)	25	25	25	25	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-115	CIP (5)	25	26	26	26	Sensitive
	K (30)	23	24	24	24	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	7	7	7	7	Resistant
	IPM (10)	40	31	31	34	Sensitive
	TE (30)	21	22	22	22	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-99	CIP (5)	30	30	30	30	Sensitive

	K (30)	15	15	15	15	Resistant
	CFM (5)	0	0	0	0.0	Resistant
	ATM (30)	0	0	0	0.0	Resistant
	FEP (30)	0	0	0	0.0	Resistant
	AMP (10)	0	0	0	0.0	Resistant
	IPM (10)	35	33	35	34.3	Sensitive
	TE (30)	17	20	20	19.0	Sensitive
	AMC (30)	0	0	0	0.0	Resistant
ADNEC-89	CIP (5)	27	25	26	26.0	Sensitive
	K (30)	16	15	15	15	Intermediate
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	33	35	33	Sensitive
	TE (30)	17	17	18	17.3	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-93	CIP (5)	27	27	25	26.3	Sensitive
	K (30)	16	15	17	16	Intermediate
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	28	28	31	29	Resistant
	TE (30)	16	15	16	16	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-95	CIP (5)	22	21	22	22	Intermediate
	K (30)	14	14	14	14	Intermediate
	CFM (5)	0.0	0.0	0.0	0	Resistant
	ATM (30)	18	18	17	18	Intermediate
	FEP (30)	18	18	17	18	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	18	18	30	22	Intermediate
	TE (30)	0	0	0	0	Resistant
	AMC (30)	16	17	18	17	Intermediate
ADNEC-97	CIP (5)	0	0	0	0	Resistant

Table 2: AST Results of *K. pneumoniae* in Non-NEC (BAP Medium, n=11)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
	K (30)	20	20	20	20	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	33	33	33	33	Sensitive
	TE (30)	21	22	20	21	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC 138	CIP (5)	30	30	29	30	Sensitive
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	10	10	10	10	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	31	32	31	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-132	CIP (5)	15	15	15	15	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	15	15	15	15	Resistant
	FEP (30)	18	19	20	19	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	34	35	37	35	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	11	12	10	11	Resistant
ADNEC-134	CIP (5)	22	23	23	23	Intermediate
	K (30)	25	22	22	23	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	31	38	33	Sensitive
	TE (30)	22	24	22	23	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-106	CIP (5)	30	29	30	30	Sensitive
	K (30)	22	23	24	23	Sensitive
	CFM (5)	0	0	0	0	Resistant

ADNEC-114	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	35	30	30	32	Sensitive
	TE (30)	21	21	21	21	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	25	24	25	25	Resistant
ADNEC-102	K (30)	25	25	25	25	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	35	35	33	Sensitive
	TE (30)	24	25	25	25	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	30	30	30	30	Sensitive
ADNEC-136	K (30)	23	24	24	24	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	36	35	30	34	Sensitive
	TE (30)	21	20	21	21	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	29	28	29	29	Sensitive
ADNEC-104	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	21	20	20	20	Resistant
	TE (30)	20	20	20	20	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	0	0	0	0	Resistant
ADNEC-78	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	17	17	17	17	Resistant
	FEP (30)	15	15	15	15	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	20	20	20	20	Intermediate
	TE (30)	0	0	0	0	Resistant

	AMC (30)	0	0	0	0	Resistant
	CIP (5)	22	22	22	22	Intermediate
	K (30)	16	16	17	16	Intermediate
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	38	31	34	34	Resistant
	TE (30)	16	17	15	16	Intermediate
	AMC (30)	0	0	0	0	Resistant
ADNEC-88	CIP (5)	21	26	26	24	Sensitive
	K (30)	16	17	16	16	Intermediate
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	31	32	32	32	Resistant
	TE (30)	16	17	17	17	Intermediate
	AMC (30)	0	0	0	0	Resistant
ADNEC-90	CIP (5)	26	27	26	26	Sensitive

Table 3: AST Results of *K. pneumoniae* in Suspected NEC (UTI Medium, n=15)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	35	32	36	34	Sensitive
	FEP (30)	15	16	16	16	Intermediate
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	23	22	21	22	Intermediate
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-113	CIP (5)	24	25	25	25	Intermediate
	K (30)	0	0	0	0	Resistant
	CFM (5)	18	19	19	19	Resistant
	ATM (30)	35	36	36	36	Sensitive
	FEP (30)	35	32	35	34	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	31	32	33	32	Sensitive
	TE (30)	0	0	0	0	Resistant
ADNEC-105						

	AMC (30)	11	11	11	11	Resistant
	CIP (5)	25	25	25	25	Intermediate
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	38	39	39	39	Sensitive
	FEP (30)	19	18	20	19	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	21	22	22	22	Intermediate
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-103	CIP (5)	25	26	26	26	Sensitive
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	9	9	9	9	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	32	32	32	32	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	10	10	10	10	Resistant
ADNEC-111	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	11	11	11	11	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	20	19	19	19	Resistant
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-117	CIP (5)	18	19	19	19	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	35	35	35	35	Sensitive
	FEP (30)	19	19	19	19	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	24	22	22	23	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-125	CIP (5)	25	27	27	26	Sensitive
	K (30)	24	22	22	23	Sensitive
	CFM (5)	0	0	0	0	Resistant

ADNEC-133	ATM (30)	35	35	35	35	Sensitive
	FEP (30)	36	36	36	36	Sensitive
	AMP (10)	18	18	20	19	Sensitive
	IPM (10)	30	30	30	30	Sensitive
	TE (30)	26	26	26	26	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	33	33	33	33	Sensitive
	K (30)	15	15	15	15	Intermediate
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	10	10	10	10	Resistant
	FEP (30)	17	17	17	17	Intermediate
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	32	30	32	31	Sensitive
	TE (30)	8	8	8	8	Resistant
	AMC (30)	14	14	14	14	Intermediate
ADNEC-121	CIP (5)	21	20	21	21	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	8	8	8	8	Resistant
	FEP (30)	11	11	11	11	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	19	20	20	20	Intermediate
	TE (30)	18	18	18	18	Sensitive
	AMC (30)	7	7	7	7	Resistant
ADNEC-127	CIP (5)	9	9	9	9	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	15	15	14	15	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	17	19	19	18	Resistant
	TE (30)	21	21	21	21	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-137	CIP (5)	20	21	21	21	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	12	12	12	12	Resistant

ADNEC-135

	TE (30)	19	20	20	20	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	14	14	14	14	Resistant
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-129	CIP (5)	8	8	8	8	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	11	11	11	11	Resistant
	TE (30)	21	22	22	22	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-85	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	10	10	10	10	Resistant
	FEP (30)	15	15	15	15	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	29	29	29	29	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	14	14	14	14	Intermediate
ADNEC-139	CIP (5)	23	23	23	23	Intermediate
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	10	10	10	10.0	Resistant
	FEP (30)	11	11	11	11.0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	17	18	18	17.7	Resistant
	TE (30)	12	13	13	12.7	Resistant
	AMC (30)	8	8	8	8.0	Resistant
ADNEC-101	CIP (5)	9	9	9	9.0	Resistant

Table 4: AST Results of *K. pneumoniae* in Non-NEC (UTI Medium, n=4)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
ADNEC-82	K (30)	0	0	0	0	Resistant
	CFM	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	20	20	20	20	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	21	20	22	21	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC	0	0	0	0	Resistant
	CIP	24	28	28	27	Sensitive
ADNEC-126	K (30)	0	0	0	0	Resistant
	CFM	0	0	0	0	Resistant
	ATM (30)	37	37	38	37	Sensitive
	FEP (30)	24	24	24	24	Sensitive
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	21	21	21	21	Sensitive
	TE (30)	23	23	23	23	Sensitive
	AMC	0	0	0	0	Resistant
	CIP	21	21	21	21	Sensitive
ADNEC-86	K (30)	0	0	0	0	Resistant
	CFM	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	0	0	0	0	Resistant
	TE (30)	20	20	21	20	Sensitive
	AMC	0	0	0	0	Resistant
	CIP	0	0	0	0	Resistant
ADNEC-94	K (30)	0	0	0	0	Resistant
	CFM	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	10	10	10	10	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	17	17	17	17	Resistant
	TE (30)	21	21	21	21	Sensitive

	AMC	0	0	0	0	Resistant
	CIP	16	16	16	16	Intermediate

Table 5: AST Results of *E. coli* in Suspected NEC (UTI Medium, n=7)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
ADNEC-127	K (30)	13	13	13	13	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	31	31	31	Sensitive
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	0	0	0	0	Resistant
ADNEC-113	K (30)	20	21	21	21	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	11	11	9	10	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	29	29	29	29	Sensitive
	TE (30)	24	24	24	24	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	25	25	25	25	Intermediate
ADNEC-85	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	11	11	11	11	Resistant
	TE (30)	21	22	22	22	Sensitive
	AMC (30)	0	0	0	0	Resistant
	CIP (5)	0	0	0	0	Resistant
ADNEC-87	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	9	9	9	9	Resistant
	TE (30)	21	21	21	21	Sensitive
	AMC (30)	0	0	0	0	Resistant

	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	13	15	13	13.7	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	0	0	0	0	Resistant
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-91	CIP (5)	10	11	10	10.3	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	11	11	12	11.3	Resistant
	TE (30)	15	15	15	15.0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-95	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	9	8	9	8.7	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	10	12	9	10.3	Resistant
	TE (30)	21	20	20	20.3	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-93	CIP (5)	0	0	0	0	Resistant

Table 6: AST Results of *E. coli* in Non-NEC (UTI Medium, n=3)

Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	0	0	0	0	Resistant
	TE (30)	16	16	16	16	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-76	CIP (5)	0	0	0	0	Resistant
	K (30)	0	0	0	0	Resistant
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	35	35	35	35	Sensitive
	FEP (30)	17	16	16	16.3	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	19	19	19	19	Resistant
	TE (30)	0	0	0	0	Resistant
	AMC (30)	0	0	0	0	Resistant
ADNEC-80	CIP (5)	27	28	29	28	Sensitive
	K (30)	20	20	20	20	Sensitive
	CFM (5)	0	0	0	0	Resistant
	ATM (30)	0	0	0	0	Resistant
	FEP (30)	0	0	0	0	Resistant
	AMP (10)	0	0	0	0	Resistant
	IPM (10)	30	40	35	35	Sensitive
	TE (30)	19	19	20	19	Sensitive
	AMC (30)	0	0	0	0	Resistant
ADNEC-134	CIP (5)	29	29	30	29	Sensitive