Exploring the Incidence and Gut Microbiota of Suspected Necrotizing Enterocolitis (NEC) in Neonates in Bangladesh: A Case-Control Study

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

Mathematics and Natural Sciences Department
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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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Ethics Statement

The study was conducted following the Helsinki Declaration and subsequent revisions.

Abstract

Necrotizing enterocolitis (NEC) is one of the most common and devastating intestinal inflammatory diseases in preterm infants with very low birth weights (less than 1,500 gram). Although the exact mechanisms that cause NEC remain elusive, an underlying relationship of the gut microbiome with NEC has been suggested. This study aims to compare and characterize the clinical and demographic data, gut bacteria and their antibiotic resistance pattern in suspected NEC vs. non-NEC infants. To the best of our knowledge, this is the first of this kind of study conducted in Bangladesh.

Stool samples were collected from thirty suspected NEC cases and thirty age- and sex-matched healthy controls admitted to the Ad-din Medical College Hospital. Samples were processed by traditional plating methods using both selective and non-selective culture media. Each morphologically distinct colony was isolated, and identified by the MALDI-TOF MS technique and confirmed using VITEK. The antibiogram of the identified bacteria against twelve antibiotics was performed using the disc diffusion method.

Few clinical and demographic differences were found between suspected cases and controls, however it was found that suspected NEC cases saw more abdominal changes, a higher incidence of vomiting and had almost twice as much antibiotics prescribed when compared with the control group. A higher load of bacteria of a magnitude of almost 4 times was found in the control group compared to the suspected cases in all selective and differential media. While *Escherichia coli* and *Klebsiella pneumoniae* were commonly observed in both cases and controls, the suspected NEC group showed the presence of unique bacterial species such as *Serratia marcescen*s, *Morganella*

morganii, *Kocuria kristinae*, *Streptococcus thoraltensis* and *Enteriobacter clocae*. Bacteria isolated from suspected NEC case neonates showed 100% resistance to the antibiotics Vacnomycin and Linezolid and species of *K. pneumoniae* isolated from the suspected NEC case neonates were resistant to more drugs than those isolated from the controls. Moreover, species of *M. morganii* were resistant against more than 50% of the antibiotics tested.

Keywords: - Necrotising Enterocolitis, Antimicrobial Resistance, Enteric Bacteria, Neonates, NICU, Antibiotic resistance

Dedication

To my parents and my best friend, Orpe, who believed in me and supported me during the days when I thought I couldn't make it.

- Myesha Hassan

To the parents whose babies are currently in the NICU, may Allah (SWT) ease your pain and bring good health and happiness to your family.

- Albab Maswood Haider

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Our sincere thanks also go to our Research Assistant, **Nazifa Tabassum**, whose dedication and support were unparalleled. From the very first day to the completion of every task, she stood by us like a pillar of strength. Her positivity and brightness added an incredible spark to our journey, making her an invaluable part of our experience.

Albab - Alongside the aforementioned acknowledgments, I would also like to thank the following individuals.

I would like to acknowledge and thank my parents and brother, to whom I owe everything.

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Special thanks to Orpe, who should be our third author with the amount of help she has provided us with throughout this thesis. A big shoutout to my friends from the groups dg, pain., scribbl, choas, lichu bagan and specially to Zuhaer, Ayesha and Nini.

And to Myesha, without whom I would have been completely lost and would never have been able to undertake a thesis as spectacular as this one, she deserves all the credit and even more.

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List of Acronyms

NEC	Necrotizing Enterocolitis
VLBW	Very Low Birth Weight
LBW	Low Birth Weight
TLR4	Toll-Like Receptor 4
CDC	Centers for Disease Control and Prevention
GDM	Gestational Diabetes Mellitus
SGA	Small for Gestational Age
CHD	Congenital Heart Disease
NICU	Neonatal intensive care unit
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry

Chapter 1: Introduction

1.1. Background

Necrotizing Enterocolitis (NEC) is one of the most common and severe gastrointestinal inflammatory conditions responsible for high mortality and morbidity rates amongst neonates born with a Very Low Birth Weight (VLBW) (B. K. Patel & Shah, 2012). Typically, NEC has been observed in infants that were born prematurely, but it can occasionally occur in full-term babies (*How Many Infants Are at Risk for Necrotizing Enterocolitis (NEC)*, 2024). The clinical symptoms of NEC sometimes overlap with the signs of sepsis (Rich & Dolgin, 2017), which makes the initial diagnosis challenging. Despite the challenge in diagnosis, it has been observed over the years that the incidence rate of NEC has risen; from about 150 per 10,000 live births among VLBW infants in the late 80s to approximately 800 per 10,000 live births in VLBW infants, indicating a growing threat to the health of newborns (Alsaied et al.,2020).

In Bangladesh, while neonatal care has been improving in recent years, the incidence rate of prematurely born neonates with the likelihood of developing NEC remains uninvestigated. This study aims to understand the underlying relation of gut microbiota with the likelihood of developing NEC in neonates born with a VLBW while also providing an insight about the prevalence of NEC in Bangladesh.

1.2. Pathophysiology of NEC:

The exact pathophysiology of NEC is not fully understood, but it is recognized as a multifactorial condition. The combination of genetic predisposition, intestinal immaturity, and an imbalance in

microvascular tone, alongside a strong likelihood of abnormal microbial colonization in the intestine and a highly immunoreactive intestinal mucosa, work as key causes in developing NEC. To elaborate,

- **Intestinal Immaturity:** Preterm infants are susceptible to intestinal injury, due to immature gastrointestinal functions such as immature motility, digestion, absorption, immune defenses, and barrier function (Hendrickx et al., 2019). For instance, lower secretion of gastric acid secretion can lead to NEC especially among neonates especially when further suppressed by H2 blockers (Guillet et al., 2006). Preterm infants also show an excessive inflammatory response to luminal microbial stimuli, leading to the alteration of the protective barriers in the intestine. This is further linked to increased expression of toll-like receptor 4 (TLR4) and under expression of regulatory factors like IκB, which influence inflammatory pathways. Additionally, the serum levels of several cytokines and chemokines that recruit inflammatory cells have been observed to be higher in patients with NEC than in healthy preterm infants. Among these increased cytokines, interleukin-8, which is produced by epithelial cells and mediates the migration of neutrophils to the site of inflammation and their activation, can cause necrosis and increased production of acute-phase proteins in the gut. Thus, this increase in interleukin-8 and the excessive inflammatory response produced by fetal enterocytes, contribute to tissue damage and NEC progression (De Plaen, 2013)

- **Improper Microbial Colonization:** Abnormal microbial colonization is another critical factor in NEC development. There are two factors for this - the presence of unusual microbial species and the reduction of diverse gut microbiota that occurs during the period

of prolonged antibiotic treatment. Abnormal microbial colonization is solely associated with the pathogenesis of the disease. A diverse gut microbiota generally gives protection from hospital acquired pathogens; this diversity is something NEC patients seem to lack. Consequently, preterm infants' enterocytes exhibit an excessive inflammatory response to both commensal and pathogenic bacteria due to developmental immaturity in regulatory mechanisms, such as reduced IκB expression (Patel and Lin, 2010). This immature response, coupled with the altered microbiota, is considered a key driver of NEC pathogenesis.

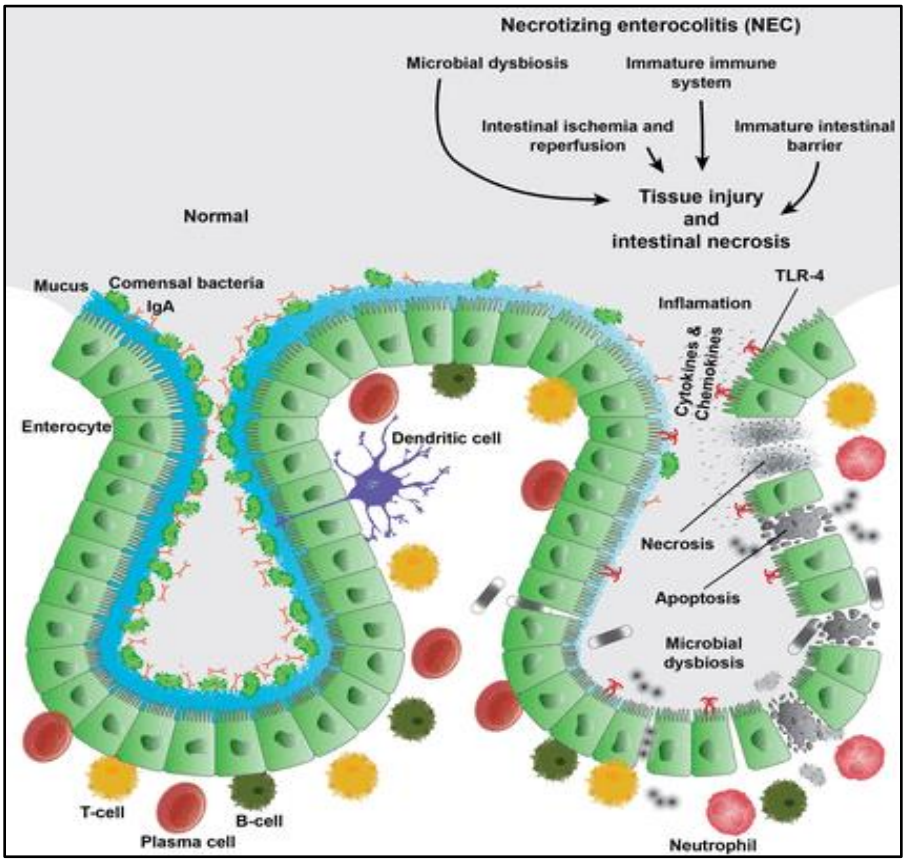

Figure 1.2: Pathophysiology of NEC (Stanikova et al., 2023)

- **Hypoxia–Ischemia:** In earlier studies, hypoxia-ischemia was considered as one of the major reasons behind the pathogenesis of NEC which, considering other factors, has since been questioned. However, this does influence the development of NEC by altering the

microvascular tone, which is responsible for the production of vascular regulators such as nitric oxide and endothelin, that are believed to contribute to the downstream pathogenic cascade leading to NEC.

Other factors like the transfusion of blood, which interferes with intestinal blood flow or hypoxia–ischemia, can play an underlying role in the pathogenesis of NEC (Neu & Walker, 2011).

1.3. Diagnosis of NEC:

A major challenge in NEC management is the absence of a definitive diagnostic method. To elaborate, even in today's date, the only diagnosis strategy that is followed while diagnosing NEC is the Bell's strategy invented in the 1970s. Bell staging system included a set of characteristics, which were used to stratify infants by illness severity, guide treatment, and support valid comparisons of the management of NEC infants into 1 of 3 stages of NEC **(Table 1.3.1 and 1.3.2).**

Table 1.3.1: 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**	**III A**	**III B**						
Reference	**1**			**3**						**20**	**17**	**19**	**22**	**24**	**25**
Risk grouping															

GA										+			+		+
Postnatal or PMA													+	+	+
Gender														+	
Ethnicity														+	
Exclusion															
SIP										+	+		+		+
Congenital anomaly													+		+
Fed <80 ml/kg/day													+		
GA>/ 36 weeks													+		+
Systematic signs															
Temp. instability	+	+	+	+	+	+	+	+	+						
Apnea	+	+	+	+	+	+	+	+	+						
Bradycardia	+	+	+	+	+	+	+	+	+						
Lethargy	+	+	+	+	+	+	+	+	+						
Acidosis (mild)								+	+	+				+	
Thrombocyt openia								+	+	+			+	+	+

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. 2: Comparison of intestinal signs and radiologic findings across NEC definitions.

Bells Criterion															
Variable Category	**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 mass							+	+	+						
Abdominal discoloration				+	+					+				+	

Radiological findings								+	+	+			+	+	+
Normal									+	+					
Ileus	+	+	+	+	+	+	+	+	+				+		
Pneumatosis		+	+			+	+	+	+	+	+	+	+	+	+
Portal Venous gas		+	+			+	+	+	+	+	+	+	+	+	+
Ascites							+	+	+					+	
Pneumoperitoneum			+						+	+	+	+			
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

But unfortunately, this strategy was not enough to represent the true etiology of NEC. As a result, after ten years, Bell's strategies were modified and classified into 3 to 6 classes. The newer staging system differentiated infants with Bell stage I by the criteria of bright red blood from the rectum (Stage IB), to those without this finding (Stage IA). In addition, Stage IIA and IIB allowed for the differentiation of the severity of illness, from infants who were mildly ill (Stage IIA) to moderately ill (Stage IIB) with ascites or portal venous gas. Finally, stage IIIB identified infants with

pneumoperitoneum, contrasting stage IIIA. However, another difficulty that has risen is the lack of sensitive and specific diagnostic biomarkers. Due to confounding factors in clinical criteria, radiographic idiosyncrasies (*e.g.*, accurately detecting pneumatosis in the intestinal wall versus fecal "bubbles") and lack of biomarkers for NEC, an accurate diagnosis is difficult to make in the absence of a pathologic intestinal specimen (R. M. Patel et al., 2020).

1.4. Risk Factors of NEC:

NEC is considered to be one of the more crucial gastrointestinal diseases that cause high mortality amongst neonates that are admitted under the Neonatal Intensive Care Unit (NICU). There are several risk factors that are responsible for developing NEC. Understanding these can give a broader sense of understanding about the disease which can also help in treating the disease. Some of the key risk factors are as followings:

1.4.1. Maternal Factors:

Though several demographic factors of motherhood like age, education, employing status or smoking are not correlated with the risk of developing NEC in the infants, there are maternal factors that have proved to be an important key risk factor in NEC. For example, it has been observed that in the case of mothers with preeclampsia, there was a 2.5 fold increased risk for NEC. Similarly, it has also been reported in a few studies that maternal chorioamnionitis increases the risk of NEC.

- **Fetal Ischemia and Growth:** Infants with intrauterine growth restriction (IUGR), with or without abnormal and small for gestational age (SGA) infants are at increased risk for NEC. Furthermore, compromising fetal blood flow before and during the delivery period can lead to fetal ischemia, thus, NEC (Rose & Patel, 2018a).

- **Types of Delivery**: In most of the cases, the early preterm babies are delivered through cesarean section. However, the association between the cesarean section delivery and likelihood of developing NEC remains unclear (Su et al., 2023a).
- **Gestational Diabetes Mellitus (GDM):** As most of the fetal nutrition comes from the mother to the fetus as a result, and as the blood sugar of the mother becomes higher because of the GDM, it seems to inhibit the blood circulation of the fetus' intestinal tract, causing ischemic necrosis of the intestinal mucosa. Following this, after birth, pathogenic microorganisms easily invade the gastrointestinal tract and colonize the damaged intestinal mucosa, causing inflammation and morbidity (Su et al., 2023a).
- **Premature Rupture of Membranes (PROM):** Another maternal risk factor is PROM that may lead to premature delivery of the infants, another leading cause of NEC 13 (Impact of Premature Rupture of Membranes on Neonatal Complications in Preterm Infants With Gestational Age <37 Weeks, 2016).

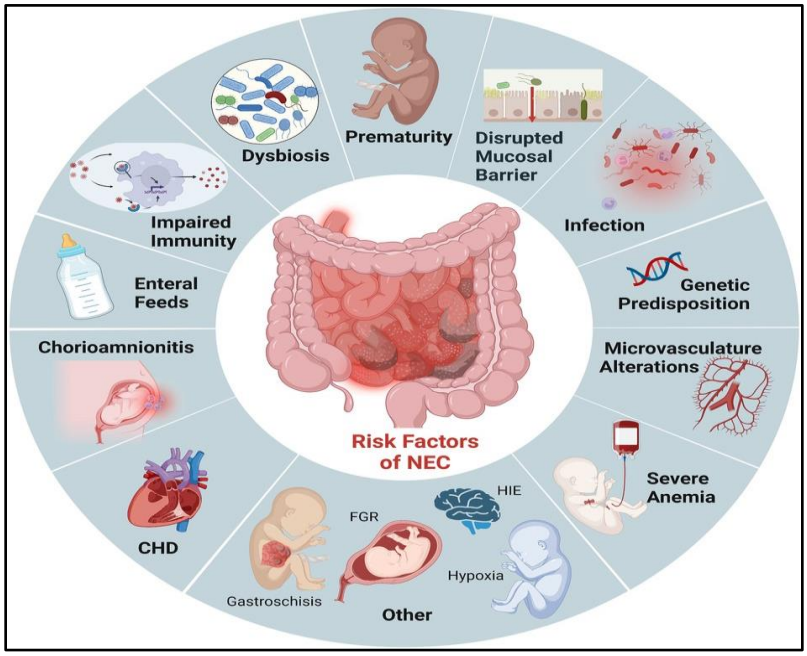

Figure 1.4.: Risk factors associated with NEC (Bautista et al., 2023). CHD: Chronic Heart Diseases: FGR: Fetal Growth Restrictions; HIE: Hypoxic Ischemic Encephalopathy.

1.4.2 Infant-Related Factors:

- **Low Birth Weight (LBW)**: LBW is the single most independent risk factor for NEC. The gut microbiota of LBW infants remains very immature, thus easily getting contact with pathogenic microorganisms can cause inflammation related to mucosal damage. On the other hand, due to the immature intestinal function and slow intestinal peristalsis of LBW infants, it proves to be a good environment for bacterial growth, leading to a large number of bacterial proliferation, leading to NEC (Bin-Nun et al., 2005).
- **Preterm Birth:** Due to the underdeveloped enteric nervous system and poor regularity of small intestinal peristalsis, premature infants are prone to excessive bacterial growth and are prone to NEC (Neu, 2007).
- **Blood Transfusion**: The possible pathogenesis of NEC is as follows: the inflammatory mediators such as TNF-α, IL-6, and Paroxysmal Atrial Fibrillation (PAF) produced during the processing of whole blood and the storage of red blood cells, and the residual white blood cells, free hemoglobin, red cell membrane fragments, etc. promote the occurrence of NEC. The pathological changes of red blood cells occurred during storage, including decreased erythrocyte deformability, increased oxygen affinity ability and decreased nitric oxide resulting in the loss of vasodilator activity, etc., resulting in the failure to improve intestinal microcirculation perfusion flow after blood transfusion; NEC may be caused by anemia.
- **Small for Gestational Age (SGA)**: SGA infants have a higher probability of NEC, neonatal asphyxia, brain injury and respiratory distress syndrome (Ree et al., 2013).
- **Other Factors:** Septicemia, congenital heart disease (CHD), respiratory distress syndrome, and pneumonia are other risk factors for NEC. In severe infection, the body

produces a variety of inflammatory transmitters which directly or indirectly cause damage to the intestinal mucosa, and then participate in the occurrence and development of NEC. In addition, in the case of sepsis, other than the direct destruction of intestinal epithelial cells, intestinal necrosis can happen by bacteria, endotoxin and other products produced by bacteria. The proportion of NEC in children with CHD is significantly higher than that in normal premature infants and neonates. Children with CHD are prone to abnormal blood distribution. The severity of necrotizing enterocolitis increases with that of respiratory distress. When respiratory distress or pneumonia occurs, the body is in an anoxic state. At this time, in order to ensure the oxygen supply of the vital organs of the child, the whole body's blood flow is redistributed, mainly the blood flow is reduced, leading to intestinal hypoperfusion, resulting in intestinal mucosa damage, thus, NEC (Impact of Premature Rupture of Membranes on Neonatal Complications in Preterm Infants With Gestational Age <37 Weeks, 2016).

1.5. Epidemiology of NEC:

NEC is responsible for 1 in 10 neonatal deaths globally (Bethell & Hall, 2023). According to the Centers for Disease Control and Prevention's (CDC) National Center for Health Statistics (NCHS) in 2019, NEC has been listed as one of the 10 leading causes of infant mortality (*How Many Infants Are at Risk for Necrotizing Enterocolitis (NEC)*, 2024). It typically occurs in the second to third weeks of the newborn's life. Along with genetic factors, several additional factors like VLBW, formula feeding can play a crucial role in developing NEC. It is estimated that globally, the incidence varies from 0.3 to 2.4 infants per 1000 live births. In the United States the incidence ranges from 1 to 7.7% of NICU admissions (Kosloske, 1994). Overall, mortality ranges from 10%

to 50%. However, in the most severe cases, involving perforation, peritonitis, and sepsis, mortality approaches 100% (Ginglen & Butki, 2023).

Multiple studies have shown NEC rates vary by region, network and country. From the previous studies, the European countries and the United States (US) seem to be highly at risk of developing NEC. The incidence rate was about 17% and 16% respectively in these two countries between the years of 2002-2016. On the other hand, Australia and Canada reported lower rates (6.4% and 9%). However, all this data also highlights that based on the regional factors, the population that remains to be at risk varies. These variations highlight the influence of regional factors on NEC risk, such as higher rates among VLBW infants in Australia and Europe versus preterm infants in the US (Alsaied et al.,2020). Additionally, racial disparities, such as higher NEC rates among Black infants in the US, have been documented.

1.5 Study Gap:

Globally, numerous reports from the World Bank have shown that there is a high prevalence of mortality amongst premature infants, particularly in Asia, including Bangladesh. This observation suggests that the incidence of NEC is also likely higher in Asia, especially in Bangladesh, but despite the global prevalence of NEC, there is a lack of NEC incident reports from Bangladesh. While Bangladesh does report a relatively high rate of low-birth-weight infants (about 23 in every 1000 babies) the specifics regarding NEC incidence and its association with mortality remain elusive (World Bank Open Data, n.d.).

Recognizing this gap, this study aims to address these issues by assessing the role of NEC as a contributing factor in the neonatal mortality rate in Bangladesh. Additionally, the study aims to explore associated risk factors to better understand and mitigate the impact of NEC on newborns.

To date, no case-control studies have investigated the mortality, incidence, and risk factors of NEC in Bangladesh, making this study a pioneering endeavor in the country.

1.6 Aims of the study:

The main aim of this study is to explore the incidence and gut microbiota of suspected necrotizing enterocolitis (NEC) in neonates in Bangladesh.

The specific objectives are as follows:

1. To determine the prevalence of NEC in Bangladesh.
2. To compare the bacterial load in the case and control group.
3. To characterize the gut microbiota of the suspected NEC and Non-NEC patients.
4. To analyze the antibiogram pattern of both groups within the context of Bangladesh.

Chapter 2: Methods and Materials

2.1 Study Design

The study was designed as a case-control study

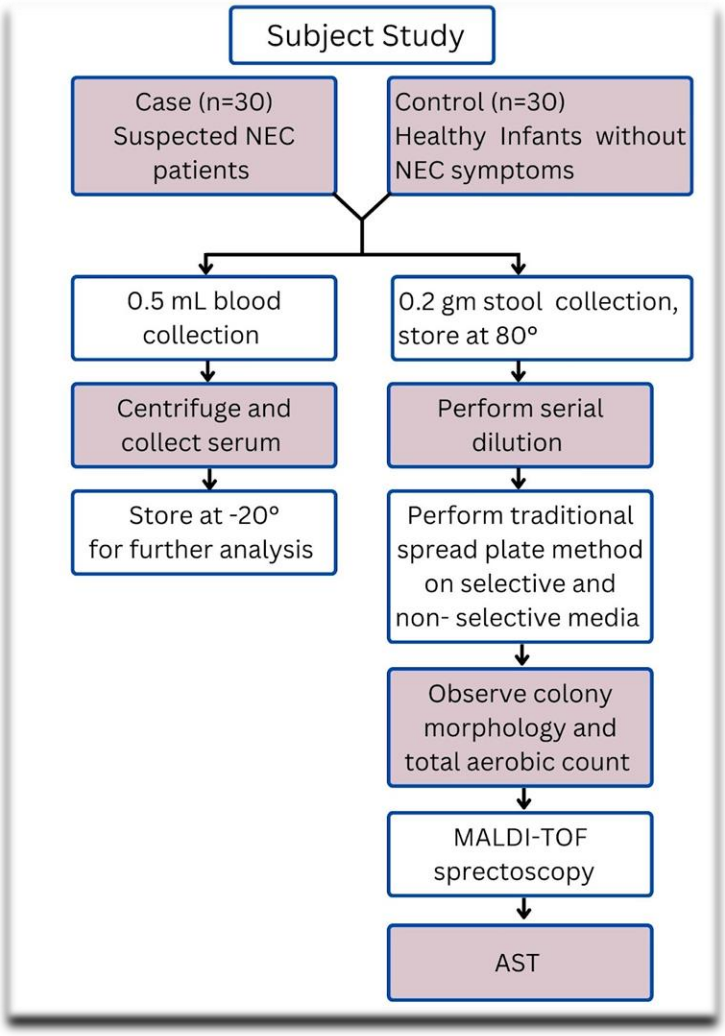

Figure 2.1: Schematic illustration of the study design

2.2 Selection of the Case and Controls:

Case: A total of 30 suspected NEC cases with strong clinical evidence i.e., abdominal distention, vomiting, low birth weight has been selected as our case. All these cases were exclusively selected from Ad-Din Medical College and hospital.

Controls: A total of 30 sex and gestational age matched healthy controls without NEC symptoms were taken from the same hospital.

Table 2.2: Inclusion and exclusion criteria

Participant	Inclusion criteria	Exclusion criteria
Cases	a) Infants admitted in the NICU b) Suspected NEC case c) Gestational Age between 28 weeks to 40 weeks d) All participants regardless of sex, socioeconomic status, and education of their parents.	a) Healthy infants outside the NICU
Control	a) Healthy Infants without NEC symptoms b) Age and sex matched to the case c) All participants regardless of sex, socioeconomic status, and education of their parents.	a) Healthy infants outside the NICU

2.3. Questionnaire:

I. **Demographic Data**: This part of the questionnaire comprises the demographic data specially, information such as the parent's name, age, educational qualification, occupation and monthly income etc. were recorded.

II. **Clinical Information**: This section comprises the clinical data of each patient, including the birth weight, length of the baby, any potential symptoms related to NEC observed in the infants immediately after the birth and admission, *i.e*, feeding intolerance, vomiting, fever, abdominal distention, any history of blood transfusion or any surgery. Additionally, the antibiotic history along with any supplements that might have been given to the infants were also recorded.

III. **Maternal History**: A brief history of the mother focusing on the type of delivery, mother's antibiotics history before and after the delivery and any recent complications that have been observed were recorded.

2.4. Consent and Ethical Issues:

The parents of the participants were fully aware of the purpose and protocol of investigating the study. Signed consent was taken from the parents of each patient party. The demographic and clinical data were directly obtained from the participant's report in the presence of attending healthcare professionals. Additionally, the maternal history was recorded using a structured questionnaire. The study was approved by the International Review Board (IRB) of the Department of Mathematics and Natural Sciences, BRAC University.

2.5 Media and Materials:

Mannitol Salt Agar (MSA), HiChrome UTI agar, Blood agar, Nutrient Agar (NA), Red-top mini collector, Stool collection containers, 0.9% NaCl buffer solution, T1N1 agar media and Glycerol

($C_3H_8O_3$) for stocking were used. Furthermore, for performing Antibiotics Susceptibility Test (AST), Muller Hinton Agar (MHA) was incorporated.

2.5.1 Blood Collection Tubes:

To collect the serum sample, red capped tubes, typically known as mini-collectors were used. These tubes do not contain any chemicals or blood clotting additives that could affect the formation of serum; the clear liquid part collected after the blood clotting takes place. These minicollectors are specifically used for serum collection.

Figure 2.5.1 : Red-top mini collectors (MedicalExpo,0.5mL)

2.5.2 Stool Container:

Stool samples were collected using blue- capped plastic containers. These do not contain any additives that may interfere with the sample.

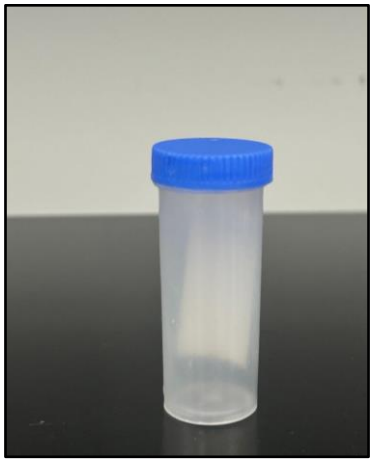

Figure 2.5.2: Stool sample collection tube

2.6 Media:

2.6.1 Mannitol Salt Agar (MSA):

MSA is both a selective and differential media. To specify, MSA contains high concentration of salt in it which only allows the selective growth of *Staphylococcus* species isolated from the clinical and nonclinical samples. This media was used to isolate *Staphylococcus* from the gut microbiota of the suspected NEC patients. It is also composed of HM peptone B and protease peptone that supply the necessary trace nutrients that allow only *Staphylococcus* to grow. However, because of its unique composition this medium also works as a differential media.

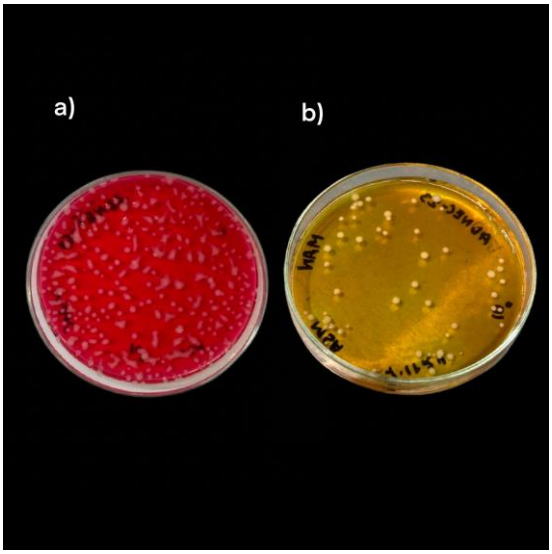

Figure 2.6.1 : (a) Mannitol Non-fermenting *Staphylococcus epidermidis* ; (b) Mannitol fermenting *Staphylococcus aureus*

To elaborate, in MSA, the key component is mannitol which works as an available carbohydrate fermenting source. Fermentation of mannitol leads to acid production which changes the color of the media. The color change is identified by the indicator present in the media, namely, phenol red. *Staphylococcus aureus* ferments the mannitol present in the media and through acid production, and thus forms yellow colonies with a yellow zone. The other group of *Staphylococcus i.e., Staphylococcus epidermidis* forms a red to pink color colony as it is unable to utilize the carbohydrate source from the media, and cannot produce acid. [ExoDiagnóstica Científica, n.d]

2.6.2 Blood Agar:

Blood agar is known as a widely used enriched media that allows the growth of typically fastidious bacteria but on the basis of the hemolysis properties of the bacteria, it can also work as a differential media.

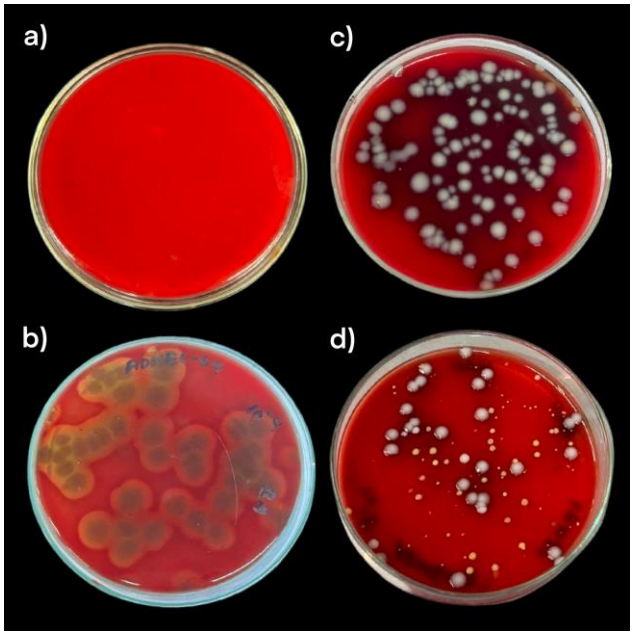

Figure 2.6.2: a) Blood Agar, b) Alpha Hemolysis c) Beta hemolysis, d) Gamma Hemolysis

To prepare this media, the blood agar base is first prepared and cooled down to 45-55 degree Celsius. After this, about 5% sterile defibrinated blood is added and swirled for a while. Prepared blood agar media is plated onto sterile plates. Similar to the MSA, stool samples from the case and controls were cultured on it. Based on the hemolysis pattern, the presence of several types of bacteria can be observed. To illustrate:

a) **Alpha hemolysis**: It is known as "**partial hemolysis**" as in case of the alpha hemolysis, hemoglobin of the red blood cell breaks down into methemoglobin in the medium surrounding the colony causing brownish or green discoloration of the medium.

b) **Beta Hemolysis**: Unlike Alpha hemolysis, Beta hemolysis causes the "**complete hemolysis**" of the red blood cells producing a clear zone surrounding the colonies. It is mostly caused because of the production of toxins by several bacterial species. For example, *Streptococcus pyogenes* produces oxygen labile toxin named "Streptolysin o"

which in presence of low level of oxygen, the hemolysin get activated causing beta hemolysis. On the other hand, the *Streptococcus agalactiae* and *Listeria monocytogenes* produce very weakly reactive toxins thus a clear zone only beneath the colonies are observed.

c) **Gamma hemolysis:** It indicates "**No clear zone**" indicating the absence of red blood cell lysis thus, no lysis at all. For instance, *Enterococcus faecalis* along with several *Streptococcus* and *Staphylococcus* species forms gamma hemolysis on the blood agar. [Buxton, 2005]

2.6.3 Hi-Chrome UTI agar:

It is a differential but non-selective media. In most of the cases, this media is used to isolate bacteria from clinical samples but it can also be used to identify bacterial load from other food and environment samples. It allows the growth of some gram-positive and gram-negative bacteria. Observing the isolated colony morphology, the bacteria can be determined. To explain, the chromogenic substrate present in the composition of the media is cleaved by the enzymes produced by several bacterial species including *Enterococcus*, *Escherichia coli* and coliforms.

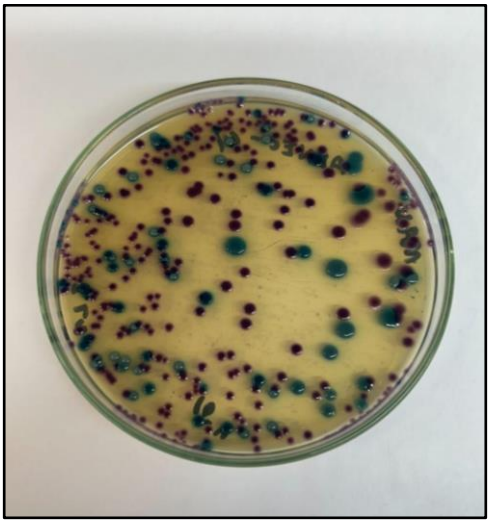

Figure 2.6.3.: Bacterial growth on HiChrome UTI agar

The presence of amino acids like phenylalanine and tryptophan from peptones help in the detection of tryptophan deaminase activity, indicating the presence of *Proteus* species, *Morganella* species and *Providencia* species. One of the chromogenic substrates is cleaved by ß-glucosidase possessed by *Enterococci* resulting in formation of blue colonies. *Escherichia coli* produce pink colonies due to the enzyme ß-D-galactosidase that cleaves the other chromogenic substrate. Colonies of Proteus, *Morganella* and *Providencia* species appear brown because of tryptophan deaminase activity. [ExoDiagnóstica Científica, 2015]

2.6.4 Nutrient Agar:

Nutrient agar is used as a non-selective, non-differential media typically used to cultivate and enumerate the total number of bacterial loads present in one sample. It is composed of beef extract, peptone and agar. Beef extract works as the key nutrition source such as carbohydrate, minerals, organic nitrogen compounds and salts whereas the peptones are the principal sources of organic nitrogen, particularly amino acids and long chained peptides. Agar is the solidifying agent. [HiMedia Laboratories. n.d]

To prepare this NA, agar is liquefied and cooled to 45-50°C and poured onto the petri dishes. Then we have to allow it to solidify for at least 30 minutes. Finally, it is prepared to obtain isolated colonies from specimens. It can also be used in case of preparing other enrichment media.

2.7 Sample collection and processing:

2.7.1 Blood Sample:

Upon receiving the consent from the parents, about 0.5 ml blood is collected from both of the cases and control which is then kept in a resting position for about 10 minutes. Following this, the rep pot has been centrifuged at 3000 rpm for another 10 minutes. Because of this centrifugation, the blood cells get settled at the bottom whereas the serum part remains as the supernatant. Finally, the serum is collected in a separate micro centrifuge tube and stored at -20°C for further immunological analysis.

2.7.2 Stool Sample:

Approximately 0.2 grams of stool samples were collected from the infants using a sterile swab and stored in a plastic container at -80°C until further processing.

2.8 Serial Dilution:

Serial dilution refers to the sequential dilution of a sample to obtain a usable concentration. Developed by Robert Koch, this method is integral to the standard plate count technique, a reliable approach for quantifying bacteria, fungi, or viruses in various samples, including water, blood, and clinical specimens. The standard plate count generally ranges from 30-300 colonies per plate. For performing serial dilution, firstly prepare the initial sample solution by mixing approximately 5 ml of NaCl solution with the stool sample. Further, 6 more test tubes containing 9 ml NaCl solution are prepared and labelled (10^{-1}, 10^{-2}, 10^{-3}…) accordingly. From the main sample then 1 ml diluted stool sample is transferred to the first test tube labelled as 10^{-1} and vortexed properly. This ensures a proper mixture of the sample. Repeat this process sequentially for all test tubes until the last dilution is achieved. After completing the serial dilution, 0.1 µL of diluted samples from each test tube are plated onto the selected media and incubated overnight to observe the growth. This

technique is a widely adopted technique specifically used for CFU count. The CFU count is determined using the formula:

CFU/mL= (Number of colonies×Dilution factor)/Volume plated (mL)

If the colony ranges > 300, it is regarded as too numerous to count (TNTC) while if the CFU <30, it is regarded as Too Few To Count (TFTC).

2.9 Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS):

MALDI-TOF MS, is a rapid molecular tool for identifying and diagnosing bacteria from clinical samples. In this process, intact cells or the extraction of the cell is used to identify the unknown bacteria. Unlike the conventional diagnosing technique, MALDI-TOF proves to be more accurate, less time and labor consuming. It has a wide range of usage including microbial identification and strain typing, epidemiological studies, detection of water- and food-borne pathogens, detection of antibiotic resistance and detection of blood and urinary tract pathogens etc. For analyzing the sample, the first and foremost step is to prepare the sample within the matrix, an energy-absorbent, aromatic organic compound that donates protons to the analytes. After preparing the sample within the matrix, it is then ionized in an automated mode with a laser beam. Desorption and ionization with the laser beam generate singly protonated ions from analytes in the sample.

The protonated ions are then accelerated at a fixed potential, where these separate from each other on the basis of their mass-to-charge ratio (m/z). The charged analytes are then detected and measured using different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers etc. During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. Based on the TOF information, a characteristic spectrum called peptide mass fingerprint (PMF) is

generated for analytes in the sample. Following this, identification of microbes by MALDI-TOF MS is done by either comparing the PMF of unknown organisms with the PMFs contained in the database (UNIPORT), or by matching the masses of biomarkers of unknown organisms with the proteome database (Singhal et al., 2015).

2.9.1. Protocol:

An isolated colony from each sample is smeared over the matrix plate (MSP 96 target) and allowed it to dry adequately. This is a crucial step in order to ensure accurate sample analysis. After ensuring proper drying, 1 μL of 70% formic is added on the smeared sample which causes the cell to rupture. Following this, α-cyano-4-hydroxycinnamic acid (HCCA) of 1 μL is added which directly gets attached to the proteins of the targeted organism.

Figure 2.9.1 : Matrix plate (MSP 96 target)

Finally, the samples are sent for analysis. In the Mass spectrum machine, it can be divided into three parts (A), the analyzer which selects ions by mass-to-charge ratio (m/z) (B), and the detector that converts the ionic current into electric current (C). Bombing with a laser beam generates ions in the ionization chamber. These ions are accelerated into an electric field which directs them to

the analyzer that separates them according to their time-of-free flight (TOF: Time-Of-Flight). The smallest molecules are grasped faster than the bigger molecules.

2.10 Antibiotics Susceptibility Test (AST):

Antibiotics Susceptibility test (AST) is an in vitro process that is used widely to assess the effectiveness of an antibiotic against one microbe. There are several methods to perform AST, but one of the widely known qualitative approaches is the Kirby-Baur disc diffusion method where antibiotics disc or paper discs are placed onto the pathogenic bacteria grown on the Mueller Hinton Agar (MHA) plates. MHA is chosen for the following purposes- a) The presence of starch in its composition- it absorbs any toxic materials that might be produced b) Greater diffusion rate- facilitates the diffusion of the antibiotic disc c) it is a non-selective media meaning, all the fastidious and non-fastidious organism can grow in it (Hudzicki, 2009).

After a proper incubation period, observing the presence or absence of a zone around the disc indicates sensitivity or resistance toward one antibiotic. For this project, 12 different antibiotics were used to observe the susceptibility pattern of the microbes against the antibiotics.

2.10.1. Preparation of the Inoculum:

The inoculum was standardized before further testing. In brief, , a sterile loop was used to collect isolated colonies from the fresh sub-cultured NA media and inoculate to prepare 10 ml NaCl solution in sterile test tubes and vortex to prepare a homogeneous mixture. The turbidity was adjusted by comparing the inoculum solution with the 0.5 McFarland standard (~ 1-1.5x 10^8 CFU/mL) solution. If the optical density of the inoculum were not in between 0.08-0.1, then the inoculum was adjusted by further adding or diluting the solution.

2.10.2. Inoculation of the MHA Plate:

Further, dip one sterile swab onto the inoculum solution and rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. Spread the swab evenly

across the dried surface of the MHA plate by streaking three times, rotating the plate approximately 60 degrees between streaks to ensure uniform distribution. Leave the lid of the plate slightly ajar, allowing the plate to sit at room temperature for 3 to 5 minutes.

2.10.3. Placement of Antibiotics Disc:

Finally, using sterile forceps, place the antibiotics disc onto the MHA plates and firmly press the plunger once to dispense the disks onto the surface of the plate. Continue to place one disk at a time onto the agar surface until all 12 disks have been placed as directed. Incubate the plates at 37°C for 24 hours and observe the presence or absence of any zone that may appear.

Table 2.10.1: 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.	Cefepime	Cephalosporin	CPM	5
5.	Vancomycin	Glycopeptide	VA	30
6.	Ampicillin	β-lactam	AMP	10

7.	Imipenem	Carbapenem	IPM	10
8.	Azithromycin	Macrolide	AZM	30
9.	Linezolid	Oxazolidinones	LZD	30
10.	Tetracycline	Tetracycline	TE	30
11.	Amoxicillin	Penicillin (beta-lactamase inhibitors)	AMC	30
12.	Ciprofloxacin	Fluoroquinolones	CIP	5

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Chapter 3: Results

3.1 Demographic and Clinical Data

For this study, the clinical samples of 30 suspected NEC neonates and gender and 30 Gestational Age matched controls were taken. Interestingly, one of the NEC neonates whose sample was taken for the study was previously found to be a control, however, over the course of the sample collection, the neonate seemed to have developed NEC, bringing our overall sample size to 59 (30 NEC, 29 control).

Table 3.1.a: Demographic Data

Characteristics		Case	Control
Gender	Male	16 (53.33%)	16 (55.17%)
	Female	14 (46.67%)	13 (44.83%)
	Total	**30 (100%)**	**29 (100%)**
Weight (kg)	0.8 - 1.29	6 (20.00%)	8 (27.59%)
	1.3 - 1.79	13 (43.33%)	6 (20.69%)
	1.8 - 2.29	5 (16.67%)	8 (27.59%)
	2.3 - 2.79	4 (13.33%)	2 (6.90%)
	2.8+	2 (6.67%)	5 (17.24%)
	Total	**30 (100%)**	**29 (100%)**
Average Weight		1.703 kg	1.952 kg

Gestational Age	Gestational Age Range (in weeks)	Case	Control
	28 - 32	7 (23.33%)	10 (34.48%)
	33 - 36	18 (60.00%)	14 (48.28%)
	37 - 40	5 (16.67%)	5 (17.24%)
	Total	**30 (100%)**	**29 (100%)**
Average Gestational Age		35 weeks	34 weeks
Age Range of Mothers	Mothers Age Range (in years)	Case	Control
	18 - 23	9 (30.0%)	9 (31.03%)
	24 - 29	15 50.0%)	11 (37.93%)
	30 - 35	3 (10.0%)	6 (20.69%)
	35+	1 (3.33%)	2 (6.90%)
	Not Reported	2 (6.67%)	1 (3.45%)
	Total	**30 (100%)**	**29 (100%)**
Average Age of Mother		24.85 years	26.5 years

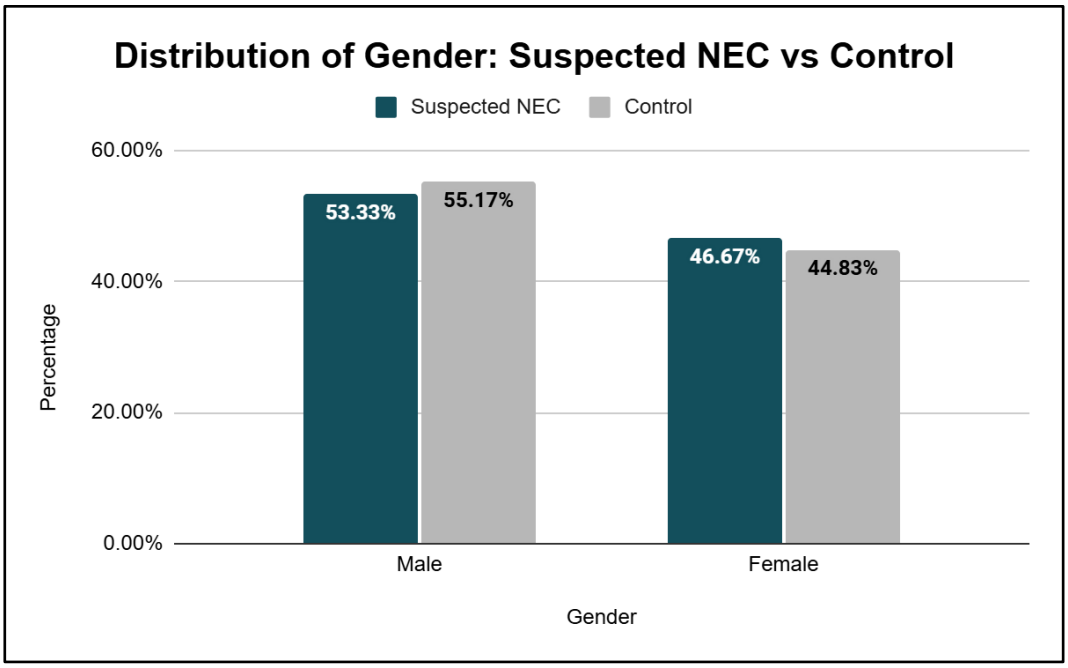

Fig 3.1a: Distribution of Gender in Percentage: Suspected NEC vs Control

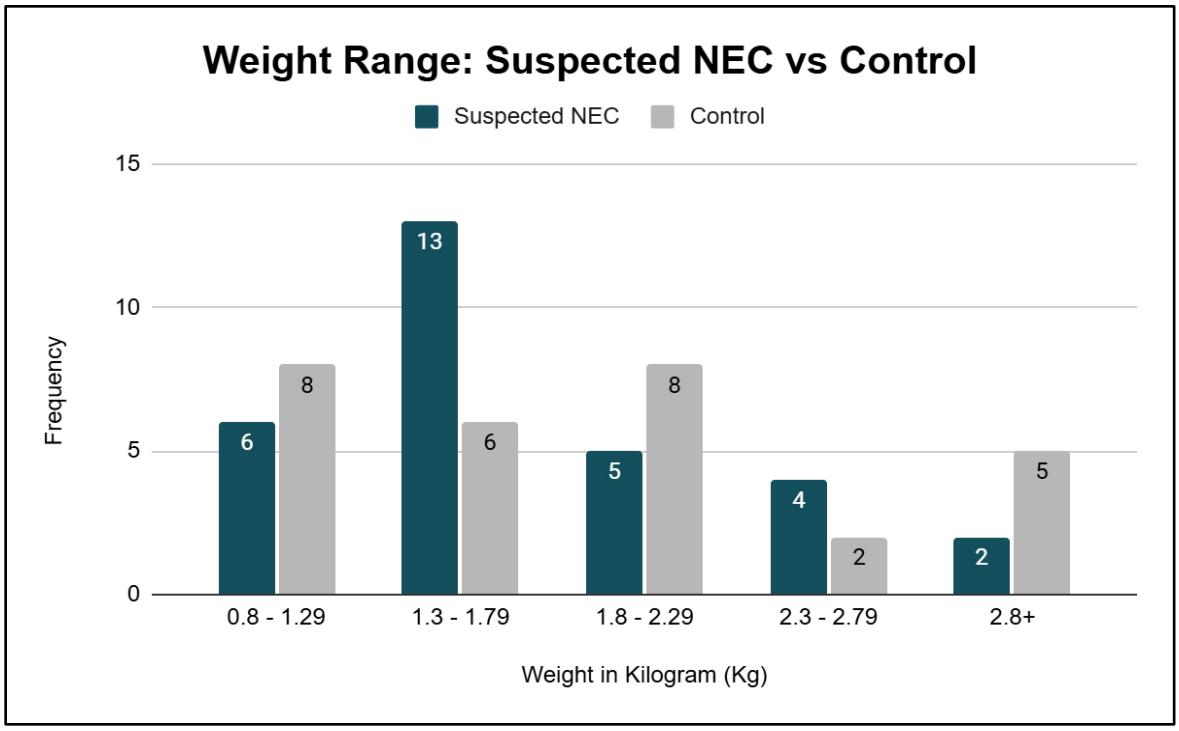

Fig 3.1b Weight Range: Suspected NEC vs Control

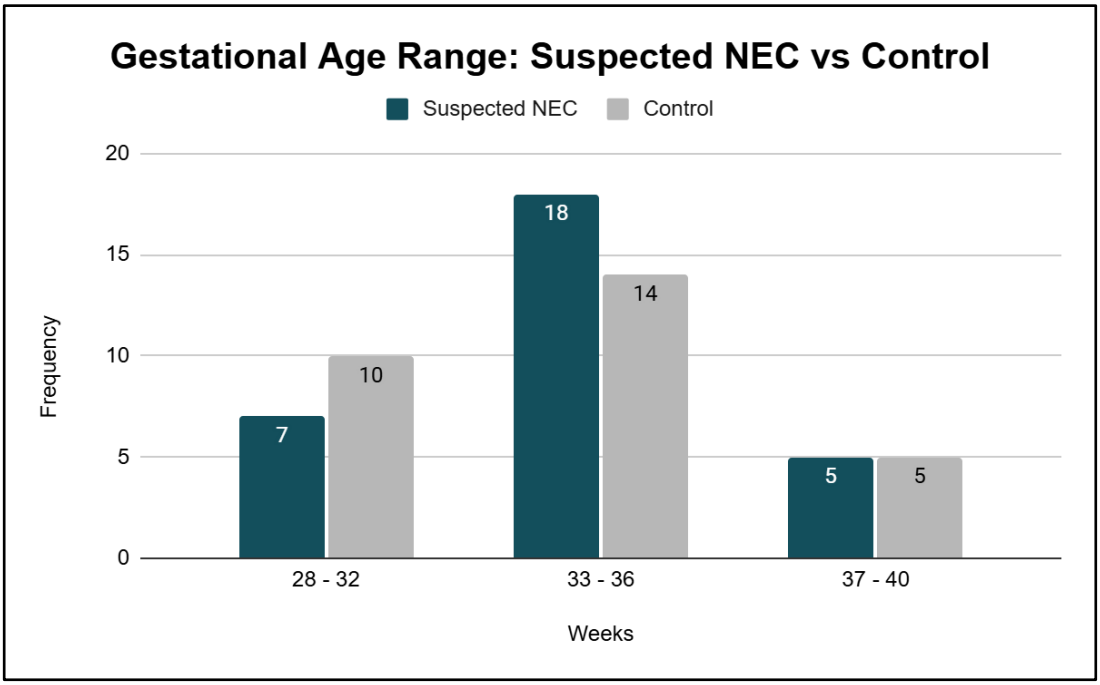

Fig 3.1c Gestational Age Range in weeks: Suspected NEC vs Control

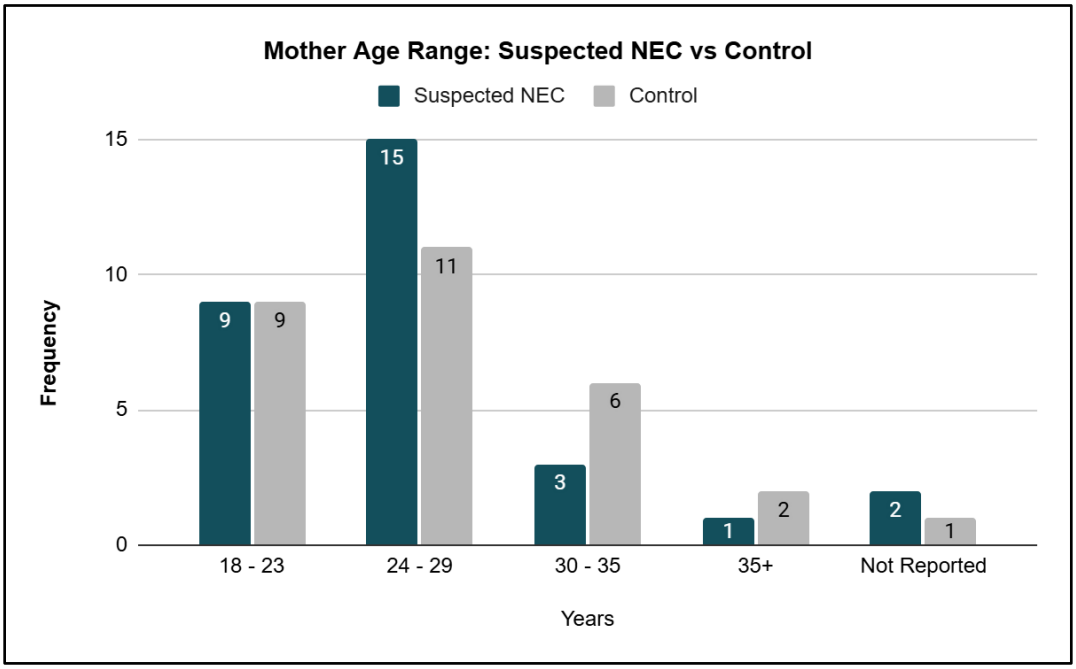

Fig 3.1d: Age Range of Mothers

The demographic data for 30 suspected NEC neonates and 29 control neonates along with their gender, birth weight range, gestational age range, has been laid out in table 3.1a, and shows that out of the 30 suspected NEC neonates, 16 (53.33%) were male and 14 (42.67%) were female, and of the 29 control neonates, 16 (55.17%) were male and 13 (44.83%) were female. The average gestational age for NEC neonates was 35 weeks and the average birth weight was 1.5 kg. In comparison, the control group reported a lower average gestational age of 34 weeks, but a higher average birth weight of 1.8 kg. The average age of the mothers of NEC neonates was 24.85 years in comparison to the control group where the average age was 26.5 years. The full list of weight, gestational age and maternal age can be found in the appendix listed under table 5.3.

The data tabulated in table 3.1a has been compared and graphically illustrated through figures 3.1a through 3.1d.

Table 3.1b: Clinical Data

Characteristic		Case (n=30)	Control (n=29)
Frequency of neonates facing feeding difficulties		16 (53.3%)	13 (44.8%)
Method of Feeding	Formula Feeding	15 (50.0%)	17 (58.62%)
	Breast Feeding	1 (3.33%)	6 (20.69%)
	Both	14 (46.67%)	6 (20.69%)
Frequency of observed abdominal changes		16 (53.3%)	0 (0%)
Frequency of neonates found to have vomited		16 (53.3%)	2 (6.9%)
Number of Antibiotics Prescribed in total for each group		122	62
Frequency of the prescription of an Antibiotic	Generic Name of Antibiotic	Case	Control
	Netilmicin	22 (73.3%)	7 (24.1%)
	Meropenem	15 (50.0%)	1 (3.4%)
	Imipenem	1 (3.3%)	13 (44.8%)
	Gentamicin	2 (6.7%)	12 (41.4%)

	Moxifloxacin	17 (56.7%)	9 (31.0%)
	Vancomycin	15 (50.0%)	4 (13.8%)
	Erythromycin	6 (20.0%)	0 (0%)
	Clindamycin	12 (40.0%)	0 (0%)
	Piperacillin	3 (10%)	1 (3.4%)
	Metronidazole	5 (16.7%)	0 (0%)
	Colistin	14 (46.7%)	7 (24.1%)
	Fosfomycin	1 (3.3%)	5 (17.2%)
	Levofloxacin	8 (26.7%)	3 (10.3%)
	Aztreonam	1 (3.3%)	0 (0%)

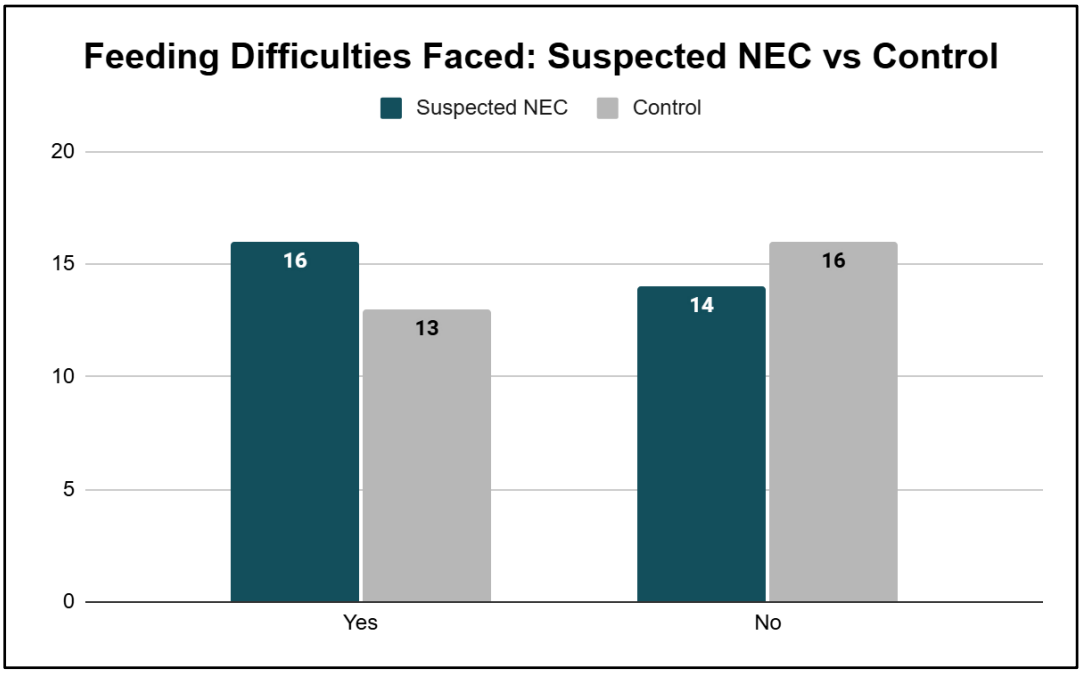

Fig 3.1e Feeding Difficulties faced: Suspected NEC vs Control

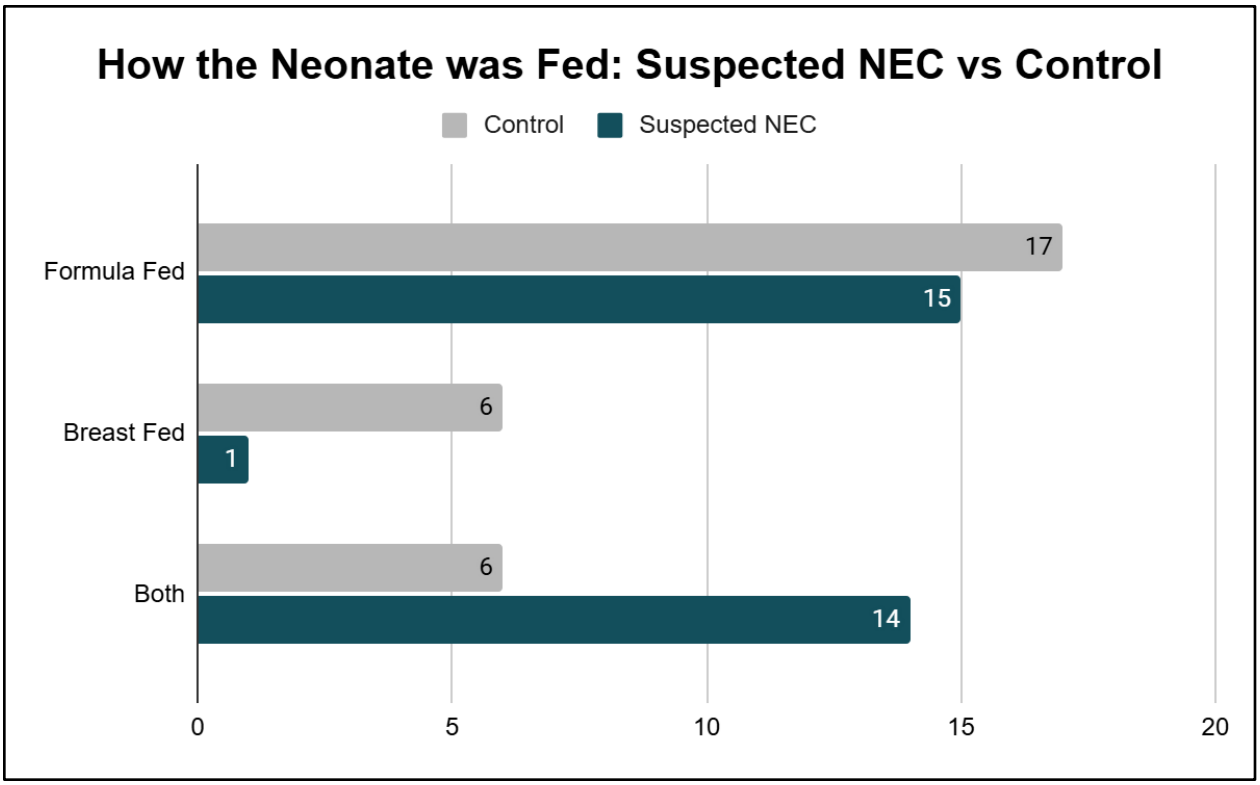

Fig 3.1f How the Neonate was Fed: Suspected NEC vs Control

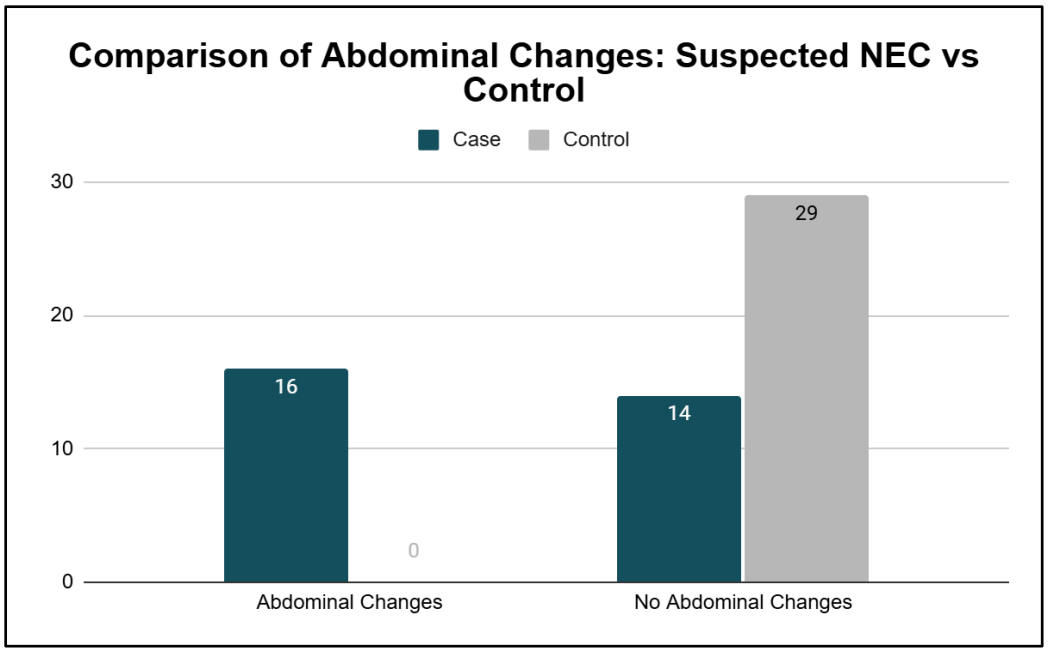

Fig 3.1g Observed Abdominal Distention

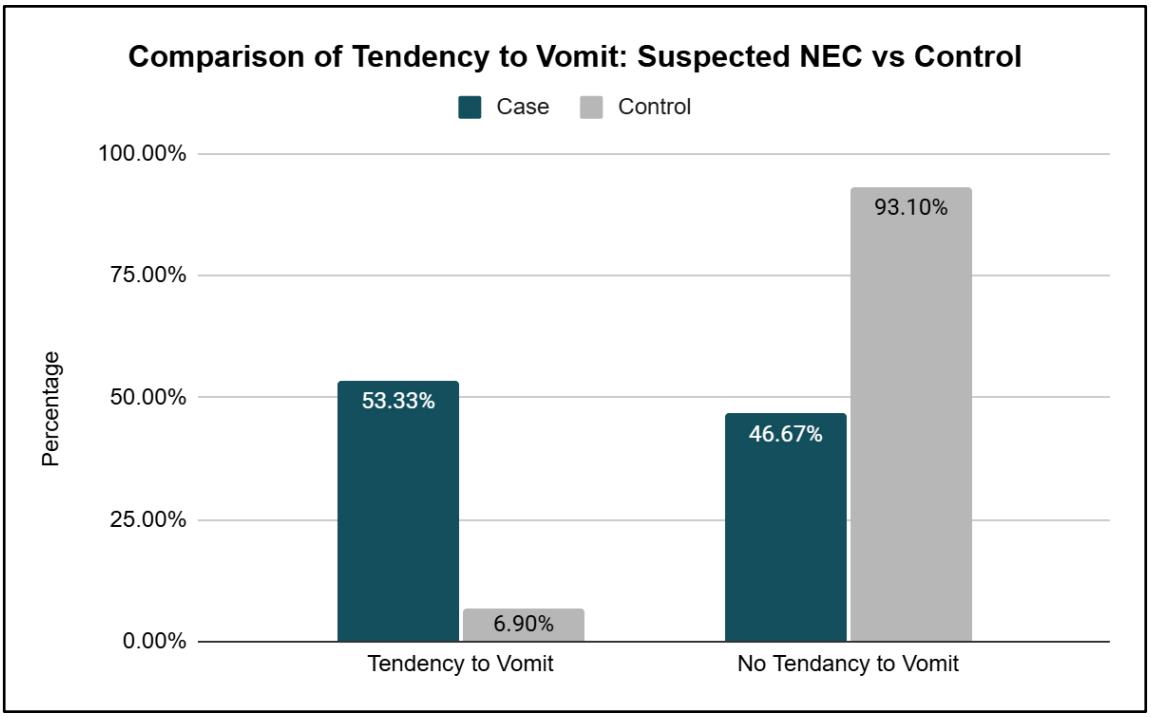

Fig 3.1h: Comparison of Tendency to Vomit: Suspected NEC vs Control

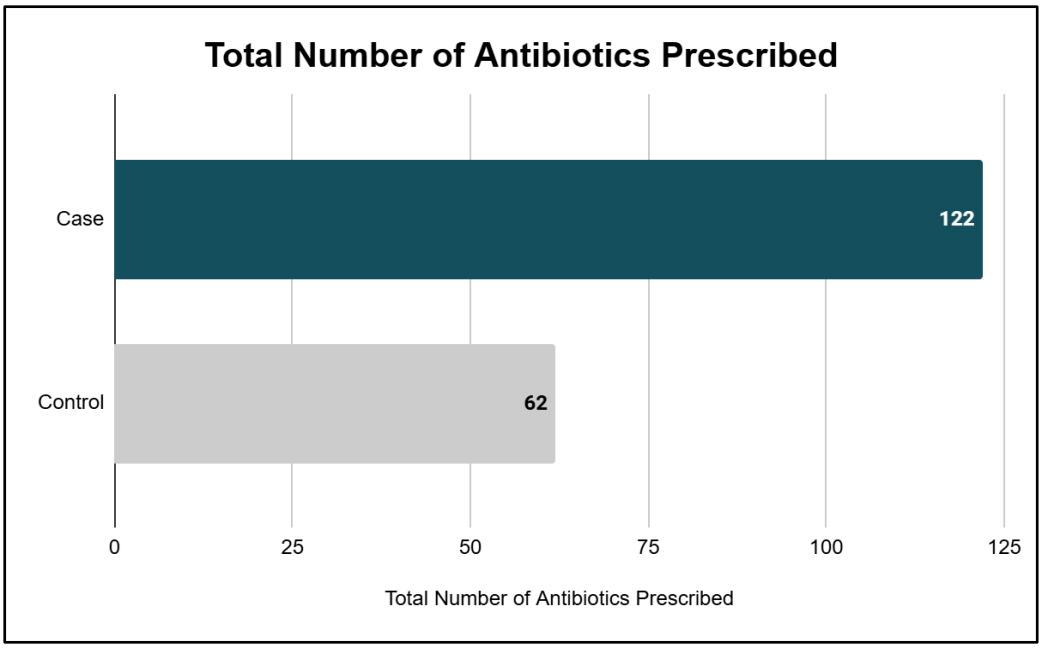

Fig 3.1i Total Number of Antibiotics prescribed: Case vs Control

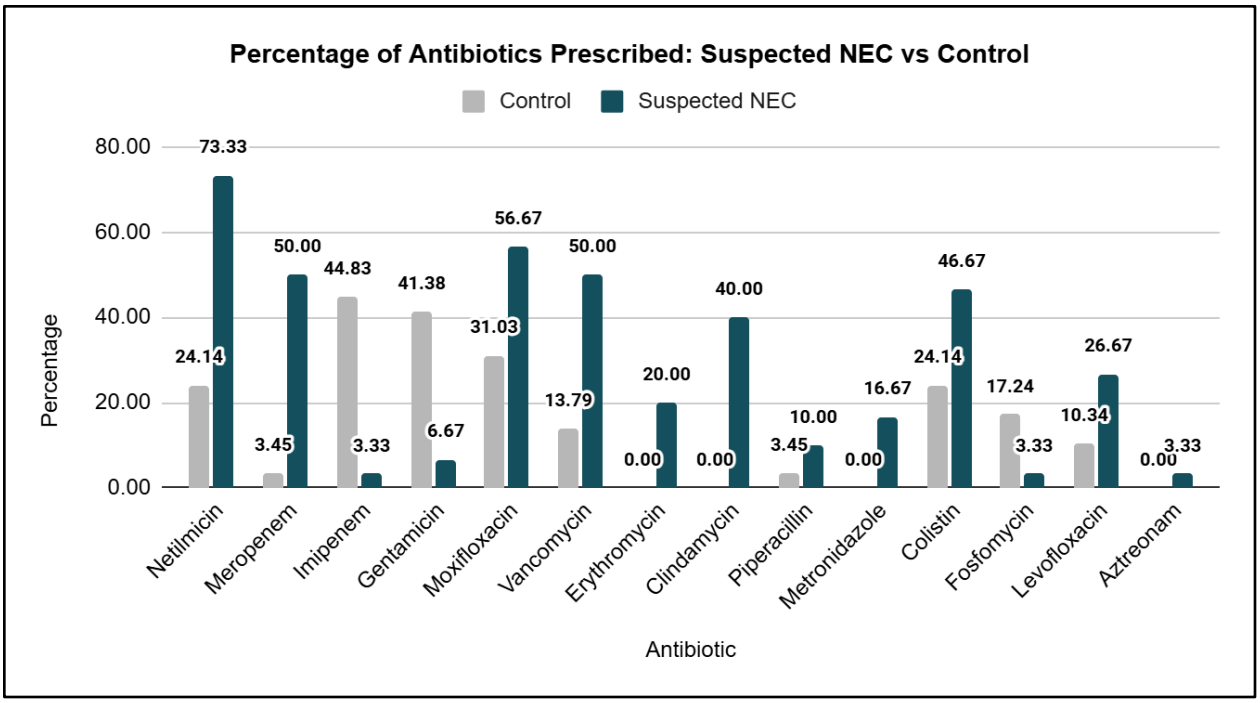

Fig 3.1j Percentage of neonates prescribed a specific antibiotic: Suspected NEC vs Control

Aside from demographic data, clinical information such as vomiting tendencies, abdominal changes, how the neonate was fed and if it experienced any feeding difficulties was also recorded, along with the number and type of antibiotics prescribed. All the data was tabulated and compiled in table 3.1b. It was found that suspected NEC neonates faced more difficulties in feeding when compared to the control group, with 16 (53.3%) neonates feeding difficulties compared to 13 (44.8%) controls facing feeding difficulties. In terms of how the neonates were fed, the suspected NEC group reported 15 (50%) neonates being fed solely through formula feeding while 14 (46.67%) neonates were fed by both formula feeding and breastfeeding, with only 1 (3.33%) neonate being fed solely by breastfeeding. The control group reported 17 (58.62%) neonates being fed solely through formula feeding, 6 (20.69%) neonates being fed solely through breastfeeding and 6 (20.69%) neonates were both formula fed and breastfed. Changes in the abdominal region, such as distention or colour changes were also recorded, with 16 (53.3%) of the suspected NEC

neonates reporting some type of abdominal change, while the control group did not report any changes in the abdominal region. The number of times a neonate vomited was also recorded, and for the purpose of this study, this distinction was simplified to show whether or not a neonate vomited at least once during its admission in the NICU. In the suspected NEC group, it was found that 16 (53.3%) neonates vomited at least once while in the NICU, and in the control group, it was found that 2 (6.9%) neonates vomited at least once while in the NICU. The number of antibiotics prescribed was also noted down during sample collection. The total number of antibiotics prescribed to the suspected NEC group was 122 and the total number of antibiotics prescribed to the control group was 62. The most subscribed antibiotic in the suspected NEC group was Netilmicin, with 22 (73.3%) neonates being prescribed it, followed by Moxifloxacin with 17 (56.7%) of neonates being prescribed it and Vancomycin and Meropenem, with both antibiotics being prescribed to 15 (50%) neonates. For the control group, the most subscribed antibiotic was Imipenem with 13 (44.8%) neonates being prescribed it, followed by Gentamicin, with 12 (41.4%) neonates and then Moxifloxacin with 9 (31.0%) neonates being prescribed it.

The data tabulated in table 3.1b has been compared and graphically illustrated through figures 3.1e through 3.1j.

3.2 CFU Count of Samples

Processing of the stool samples was carried out following the aforementioned protocol for serial dilution and plating, and after incubation for 24 hours, the number of colonies was counted and the CFU calculated using the formula mentioned in the protocol.

Key for Table 3.2a and 3.2b	
	Control
	Case

Table 3.2a CFU Count of Control Cases

Sample ID	UTI CFU/5ml	MSA CFU/5ml	Blood Agar CFU/5ml	NA CFU/5ml
ADNEC - 01	13,600,000	TNTC	10,300,000	TNTC
ADNEC - 03	620,000,000	600	122,000,000	TNTC
ADNEC - 07	3,400,000,000	0	1,130,000,000	750,000,000
ADNEC - 24	170,000	100	2,000,000	100,000
ADNEC - 72	69,600,000	11,800	191,000,000	138,000,000
ADNEC - 73	100,000	0	2,100,000	0
ADNEC - 69	30,000	6,600	140,000,000	110,000,000
ADNEC - 15	0	1,500	0	0
ADNEC - 23	6,600,000	1,100	119,000,000	17,000,000
ADNEC - 28	180,000,000	36,800	TNTC	2,000,000,000
ADNEC - 30	100,000	TNTC	11,000,000	400,000
ADNEC - 32	8,200,000	23,200	244,000,000	102,000,000
ADNEC - 36	5,000,000	200	13,000,000	5,000,000
ADNEC - 70	2,370,000	160	8,000,000	7,000,000
ADNEC - 42	40,000,000	10	82,000,000	70,000,000

ADNEC - 71	10,000	0	1,000,000	0
ADNEC - 74	56,000,000	4,800	207,000,000	108,000,000
ADNEC - 48	7,280,000	70,000	57,000,000	32,000,000
ADNEC - 50	TNTC	38,800	TNTC	TNTC
ADNEC - 52	1,390,000	TNTC	11,000,000	4,000,000
ADNEC - 54	404,000,000	TNTC	TNTC	TNTC
ADNEC - 56	75,000,000	8,000	156,000,000	44,000,000
ADNEC - 58	0	0	36,000,000	0
ADNEC - 62	0	0	2,000,000	0
ADNEC - 64	88,000,000	28,100	203,000,000	157,000,000
Average CFU	207,393,750	11,037	124,881,818	168,785,714

Table 3.2a shows the total CFU for all the samples taken from the control neonates. The average CFU/ml for samples plated on UTI Hi-chrome agar was 2.07×10^8, for MSA it was 1.10×10^4, for Blood Agar it was 1.24×10^8 and for Nutrient Agar it was 1.68×10^8.

Table 3.2b CFU Count of Suspected NEC Cases

	UTI	MSA	Blood Agar	NA
ADNEC - 11	1,670,000	TNTC	12,000,000	1,400,000
ADNEC - 16	700,000	1,100	1,000,000	300,000
ADNEC - 33	550,000	10	7,000,000	2,000,000

ADNEC - 17	47,000,000	100	101,000,000	73,000,000
ADNEC - 18	1,950,000	0	1,000,000	2,000,000
ADNEC - 19	32,000,000	900	101,000,000	90,000,000
ADNEC - 20	2,650,000	TNTC	3,000,000	7,000,000
ADNEC - 21	3,510,000	50	161,000,000	141,000,000
ADNEC - 37	3,600,000	5,000	162,000,000	190,000,000
ADNEC - 27	1,000,000	0	3,000,000	1,000,000
ADNEC - 29	370,000,000	37,000	1,050,000,000	0
ADNEC - 31	5,200,000	TNTC	36,000,000	33,000,000
ADNEC - 35	660,000	TNTC	1,000,000	1,000,000
ADNEC - 39	130,000	0	20,000,000	23,000,000
ADNEC - 41	3,360,000	8,700	0	56,000,000
ADNEC - 43	2,200,000	400	153,000,000	42,000,000
ADNEC - 45	0	0	0	7,000,000
ADNEC - 47	700,000	700	44,000,000	0
ADNEC - 49	30,800,000	2,500	180,000,000	47,000,000
ADNEC - 51	42,400,000	500	75,000,000	70,000,000
ADNEC - 53	208,000,000	600	TNTC	TNTC
ADNEC - 55	432,000,000	4,100	TNTC	TNTC
ADNEC - 57	57,600,000	0	141,000,000	113,000,000

ADNEC - 61	51,600,000	3,700	350,000,000	255,000,000
ADNEC - 63	30,000,000	0	51,000,000	53,000,000
Average CFU	53,171,200	3,112	115,347,826	52,508,696

Table 3.2b shows the total CFU for all the samples taken from the suspected NEC neonates. The average CFU/ml for samples plated on UTI Hi-chrome agar was 5.31 x 10^7, for MSA it was 3.11 x 10^3, for Blood Agar it was 1.15 x 10^8 and for Nutrient Agar it was 5.25 x 10^7.

The data shown in tables 3.2a and 3.2b have been compared and graphically illustrated through figures 3.2a and 3.2b.

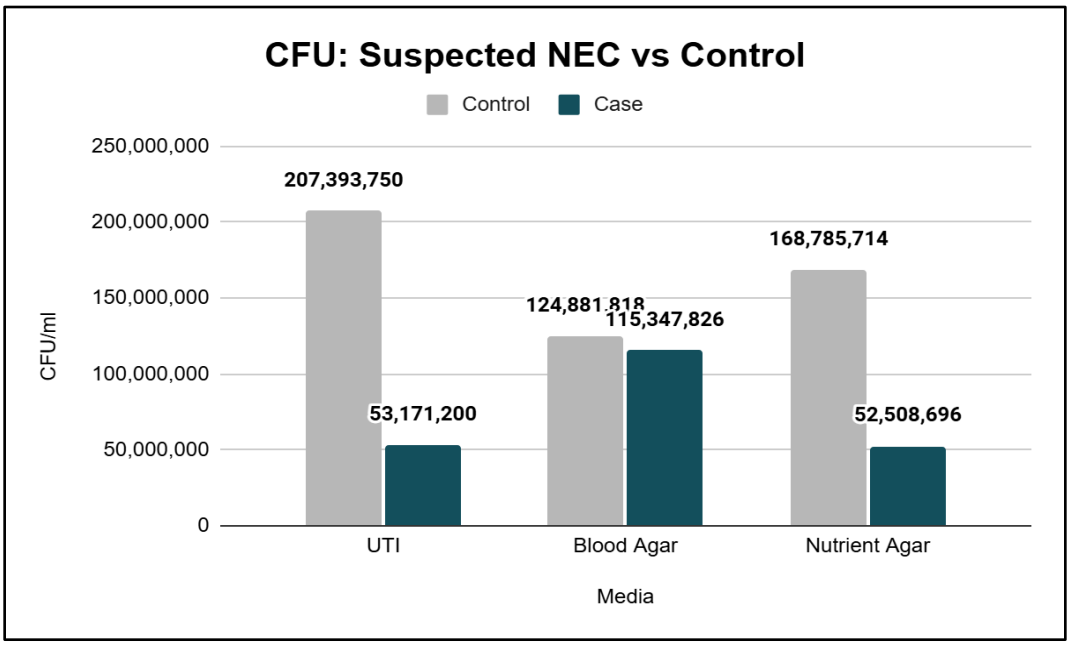

Fig 3.2a CFU Count per ml (UTI, Blood Agar, NA): Suspected NEC vs Control

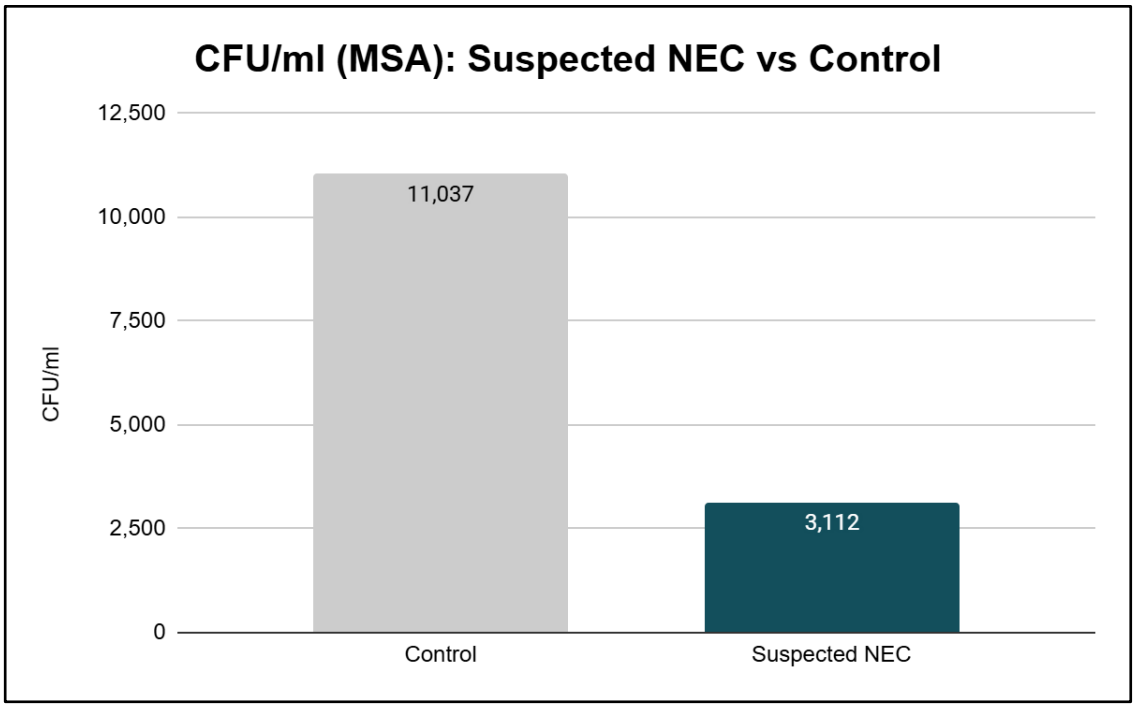

Fig 3.2b CFU Count per ml (MSA): Case vs Control

3.3 Bacterial species found in Samples

Preliminary identification of the bacteria grown post serial dilution could be carried out due to 3 of the 4 media being differential (UTI, MSA and Blood Agar) with 2 of them being both selective and differential (UTI and MSA). Until further tests such as MALDI-TOF or VITEK could be performed, the identification of the bacteria could only be labelled as suspected.

Key for Table 3.3a	
	Present
	Absent
	Control

Table 3.3a Suspected Bacterial species found in Control Cases

Sample Number	***Enterococcus faecalis***	***Escherichia coli***	***Klebsiella pneumoniae***	***Saprophyticus***	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Streptococcus agalactiae***	***Listeria monocytogenes***	**Other**

ADNEC - 01	+	+	+	+	+	-	+	-	-
ADNEC - 03	-	-	+	+	+	+	+	+	-
ADNEC - 07	+	+	+	+	-	-	+	+	+
ADNEC - 24	-	-	+	+	-	+	+	-	+
ADNEC - 72	-	+	+	+	+	+	-	-	+
ADNEC - 73	-	-	-	-	-	-	-	-	+
ADNEC - 69	-	-	-	-	-	+	+	-	+
ADNEC - 15	-	-	-	-	+	+	-	-	-
ADNEC - 23	-	-	+	+	+	-	+	-	+
ADNEC - 28	+	+	+	-	+	-	-	+	+
ADNEC - 30	-	-	+	+	+	+	-	+	+
ADNEC - 32	+	+	+	-	+	+	+	+	+
ADNEC - 36	-	-	+	-	+	+	+	-	+
ADNEC - 70	-	-	-	+	-	+	+	-	+
ADNEC - 42	+	+	-	-	+	-	+	+	-
ADNEC - 71	-	-	-	-	-	-	-	-	+
ADNEC - 74	-	+	+	-	+	-	-	+	-
ADNEC - 48	-	+	+	+	-	+	-	+	-
ADNEC - 50	+	+	-	-	+	+	-	+	+
ADNEC - 52	+	+	+	+	+	-	-	-	+

ADNEC - 54	+	+	+	-	+	+	-	-	-
ADNEC - 56	-	+	+	-	-	+	-	+	-
ADNEC - 58	-	-	-	-	-	-	+	+	+
ADNEC - 62	-	-	-	-	-	-	-	-	+
ADNEC - 64	-	+	+	+	-	+	-	+	+
Frequency of bacteria appearing in each Control	8 (27.6%)	13 (44.8%)	16 (55.2%)	11 (37.9%)	14 (48.3%)	14 (48.3%)	11 (37.9%)	12 (41.4%)	17 (58.6%)

Table 3.3a shows the suspected bacteria that were present in each of the samples obtained from the control neonates. The most common suspected organism was *Klebsiella pneumoniae* with 16 (55.2%) instances of it being suspected, followed by *Staphylococcus aureus* and *Staphylococcus epidermidis* with 14 (48.3%) instances each. Apart from the suspected organisms that could be identified due to the selective and differential nature of the media, there were 17 (58.6%) instances where bacteria that were not as commonly found in other samples were recorded or bacteria that were not detailed within the identification protocol were identified. These results have been recorded and tabulated in table 3.3c which can be found in the appendix. Where suspected bacteria could not be identified, the nature and morphology of the colony along with the dilution factor and media were elaborated upon instead.

Key for Table 3.3b	
	Present
	Absent
	Case

Table 3.3b Suspected Bacterial species found in Suspected NEC Cases

Sample Number	*Enterococcus faecalis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Saprophyticus*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus agalactiae*	*Listeria monocytogenes*	**Other**
ADNEC - 11	+	-	-	+	+	+	+	+	+
ADNEC - 16	-	-	-	+	+	+	+	-	-
ADNEC - 33	-	-	+	-	+	-	+	-	+
ADNEC - 17	+	+	-	-	+	-	-	-	-
ADNEC - 18	+	+	-	-	-	-	+	+	+
ADNEC - 19	+	-	+	-	-	+	-	-	-
ADNEC - 20	-	+	+	+	-	+	+	+	-
ADNEC - 21	-	+	+	+	-	+	+	+	-
ADNEC - 37	-	-	-	+	-	-	-	-	+
ADNEC - 27	-	+	+	-	-	-	+	-	-
ADNEC - 29	+	+	+	+	+	-	+	-	-
ADNEC - 31	+	+	+	+	+	-	+	+	-

ADNEC - 35	+	-	-	-	+	-	-	+	-
ADNEC - 39	-	+	+	-	-	-	+	-	-
ADNEC - 41	+	+	+	-	+	+	-	-	+
ADNEC - 43	-	+	+	-	-	+	-	-	+
ADNEC - 45	-	-	-	-	-	-	+	-	-
ADNEC - 47	-	-	+	-	-	+	-	+	-
ADNEC - 49	-	+	+	-	-	+	-	-	+
ADNEC - 51	+	+	-	+	+	+	+	+	-
ADNEC - 53	+	+	+	+	-	+	-	+	+
ADNEC - 55	+	+	+	-	+	+	-	+	-
ADNEC - 57	-	+	-	-	-	-	-	+	-
ADNEC - 61	-	+	+	-	+	+	-	-	+
ADNEC - 63	-	-	+	-	-	-	-	-	+
Frequency of bacteria appearing	11 (36.7%)	16 (53.3%)	16 (53.3%)	9 (30.0%)	11 (36.7%)	13 (43.3%)	12 (40.0%)	11 (36.7%)	11 (36.7%)

in each Case									

Table 3.3b shows the suspected bacteria that were present in each of the samples obtained from the suspected NEC neonates. The most common suspected organism was *Escherichia coli* and *Klebsiella pneumoniae* with 16 (53.3%) instances being suspected followed by *Staphylococcus epidermidis* with 13 (43.3%) instances being suspected and then *Streptococcus agalactiae* with 12 (40.0%) instances being suspected. Apart from the suspected organisms that could be identified due to the selective and differential nature of the media, there were 11 (36.7%) instances where bacteria that were not as commonly found in other samples were recorded or bacteria that were not detailed within the identification protocol were identified. These results have been recorded and tabulated in table 3.3c which can be found in the appendix. Where suspected bacteria could not be identified, the nature and morphology of the colony along with the dilution factor and media were elaborated upon instead.

A comparison of the suspected organisms between suspected NEC neonates and control neonates is illustrated in figure Fig 3.3.

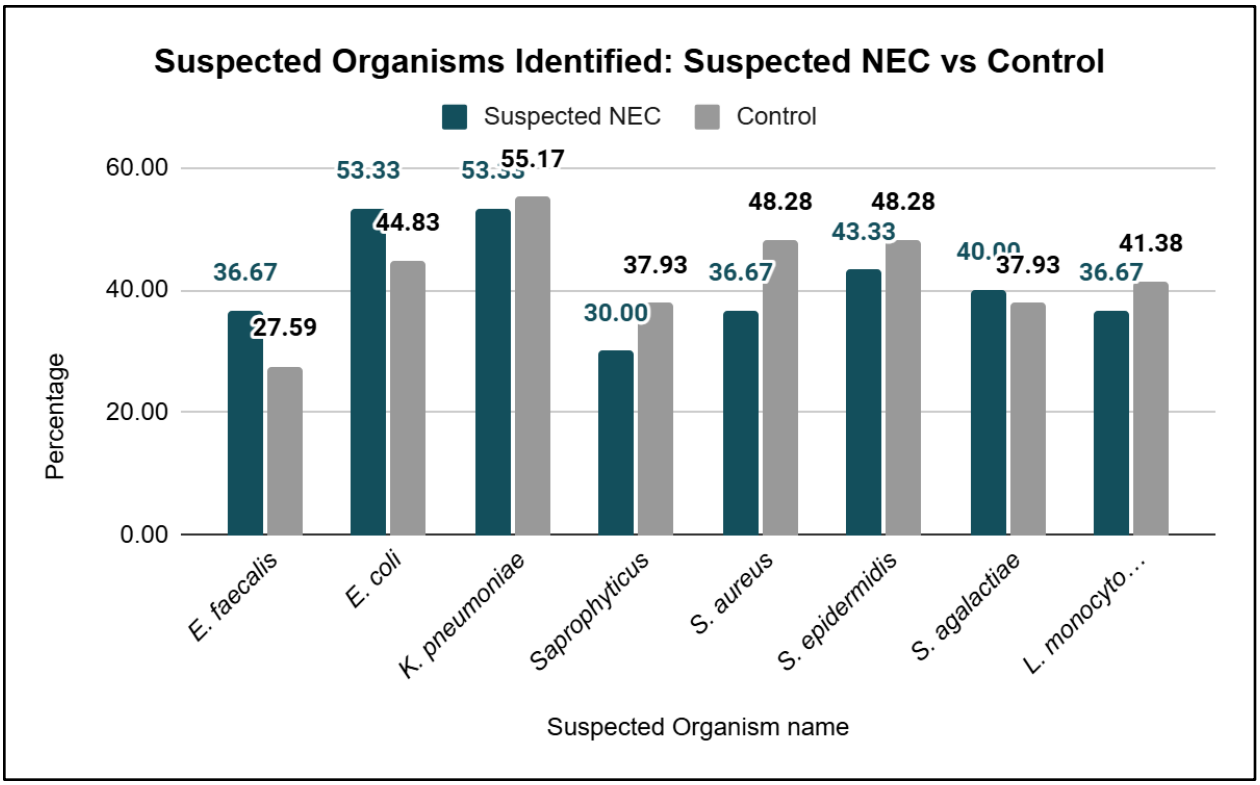

Fig 3.3 Comparison of the suspected organisms between suspected NEC neonates and control neonates

3.4 MALDI-TOF

The organisms that were identified through plating on selective and differential media could only be classified as suspected organisms. To further ensure the identity of the organisms, MALDI-TOF was carried out on 10 samples, 5 cases and 5 controls, resulting in a total of 50 organisms being identified through this process.

The full MALDI-TOF data, including what the suspected organism was and what the identified organism was, can be found in the appendix under Table 3.4 MALDI TOF.

After carrying out MALDI-TOF, it was found that 29 (54.7%) of the suspected organisms were *Klebsiella pneumoniae*, and 7 (13.2%) of the organisms were *Escherichia coli*, with a further 6

(11.3%) of the organisms unable to be identified. 5 organisms previously not identified in the protocol were identified through MALDI-TOF, the organisms being *Staphylococcus haemolyticus, Morganella morganii, Serratia marcescens, Candida tropicalis* and *Enterobacter cloacae*. The data of the total frequency of each organism is highlighted in figure Fig 3.4a

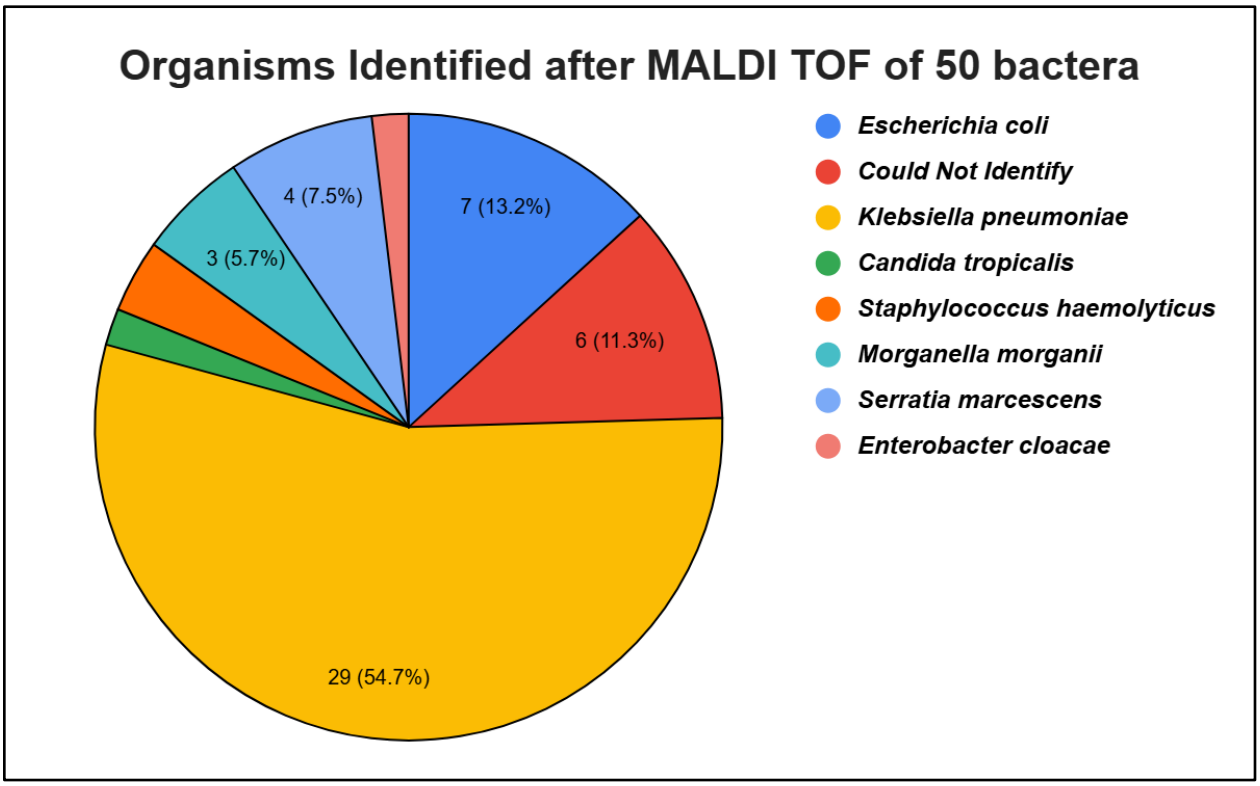

Fig 3.4a Total Count of Organisms identified after MALDI-TOF of 50 bacterial samples

When comparing the organisms between suspected NEC neonates and control neonates, it was found that the suspected NEC neonates supported 5 different organisms that the control neonates did not support. These organisms and their frequency include *Staphylococcus haemolyticus* (2), *Morganella morganii* (3), *Serratia marcescens* (4), *Candida tropicalis* (1) and *Enterobacter cloacae* (1). 5 different organisms could not be identified in the samples from the control neonates, whereas in the suspected NEC neonates, there was only 1 instance where an organism was unable

to be identified. Figure Fig 3.4b illustrates the differences in the organism counts between the suspected NEC and control neonates.

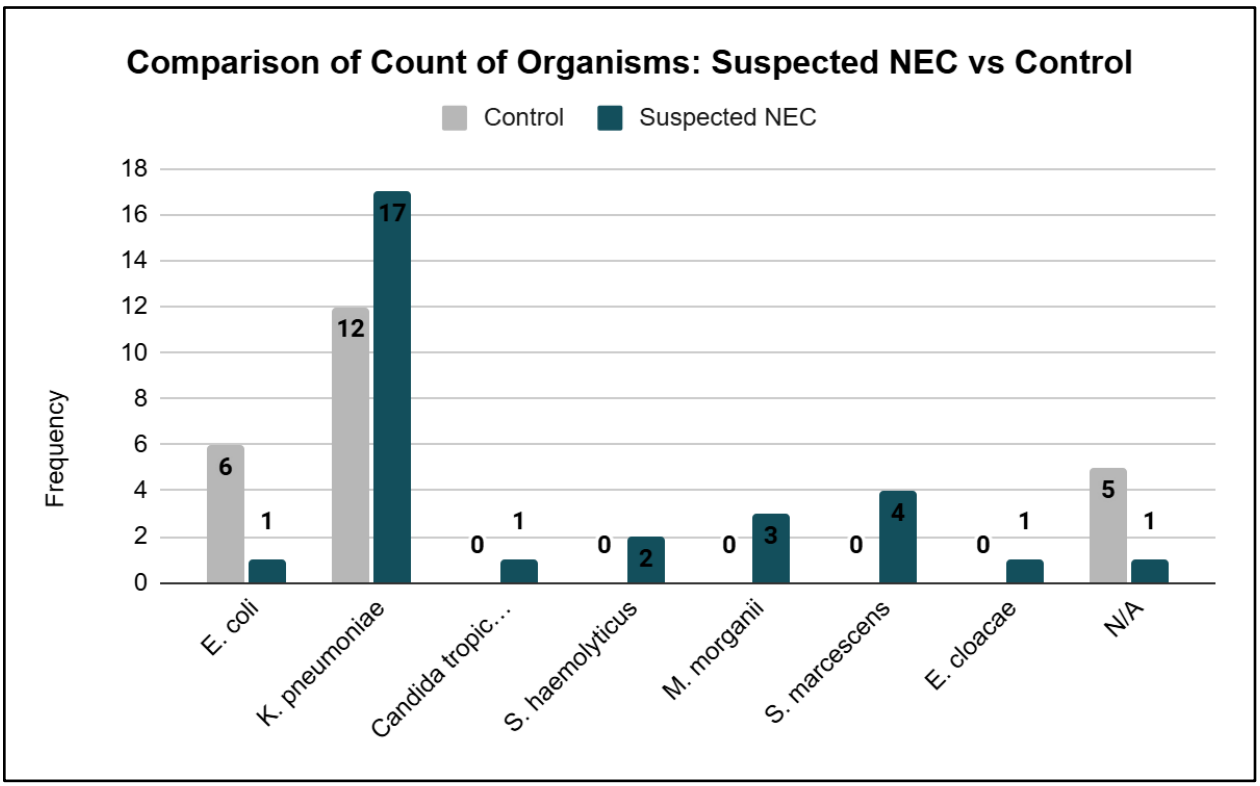

Fig 3.4b: Comparison of the count of organisms between suspected NEC neonates and control neonates. N/A: Not Applicable

3.5 VITEK

As the presumptive identification data based on differential media growth for 7 isolates did not match with the MALDI TOF data, those isolates were further checked for their identity through the use of VITEK (Table 3.5).

Key for Table 3.5	
	Control
	Case

Table 3.5 VITEK Results

Case/Control	Sample	Suspected Microorganism	VITEK Identified Microorganism
Control	ADNEC - 05	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*
	ADNEC - 05	*Staphylococcus aureus*	*Klebsiella pneumoniae ssp pneumoniae*
	ADNEC - 12	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
Case	ADNEC - 19	*Staphylococcus epidermidis*	*Kocuria kristinae*
	ADNEC - 21	*Staphylococcus epidermidis*	*Klebsiella pneumoniae ssp pneumoniae*
	ADNEC - 29	*Staphylococcus epidermidis*	*Klebsiella pneumoniae ssp pneumoniae*
	ADNEC - 20	*Staphylococcus aureus*	*Streptococcus thoraltensis*

As illustrated in Table 3.5, only 1 of the organisms matched our initial identification that was carried out through plating on selective and differential media. Out of the remaining 6 isolates, 3 (50%) were identified as *Klebsiella pneumoniae*, while the remaining isolates were identified as *Staphylococcus haemolyticus*, *Streptococcus thoraltensis* and *Kocuria kristinae*.

3.6 Antibiotic Susceptibility Test Results

Following the results from MALDI-TOF and VITEK, the isolates were prepared for Antibiotic Susceptibility Test as explained in the protocol. The zone of inhibition was measured three times and an average calculated and depending on the diameter of the zone in mm, the bacteria were classified as either resistant, intermediate resistance, or sensitive. The full list including the zones of inhibition and which bacteria exhibited what level of resistance are illustrated in table 5.6 in the appendix.

In total, 11 bacteria from suspected NEC neonate samples and 10 bacteria from control neonate samples were tested. In terms of resistance to antibiotics, samples from suspected NEC case neonates showed resistances to Vancomycin, Linezolid, Kanamycin and Ampicillin, with bacteria from suspected NEC case neonates showing 100%, 100%, 72.73% and 72.73% resistance respectively. Samples from control neonates showed resistances to Cefepime, Aztreonam, Vancomycin and Linezolid with bacteria from control neonates showing 80%, 70%, 70% and 70% resistance respectively. The total percentage of bacteria that were resistant to each antibiotic is illustrated in table 5.7 in the appendix.

If we look in terms of bacteria, we find that *Escherichia coli* and *Klebsiella pneumoniae* displayed the highest level of resistance to antibiotics. 11 isolates of *Klebsiella pneumoniae* and 6 isolates of *Escherichia coli* were tested for antibiotic susceptibility. Of the 11 isolates of *Klebsiella pneumoniae*, 6 were found in suspected NEC neonates and 5 were found in control neonates. Of the 6 *Klebsiella pneumoniae* isolated from suspected NEC neonates, 100% of them were resistant to Kanamycin, Vancomycin and Linezolid, whereas of the 5 *Klebsiella pneumoniae* isolated from controls, 100% of them were resistant to Aztreonam. Of the 6 isolates of *Escherichia coli,* 5 were isolated from control neonates, and 1 was isolated from suspected NEC neonates. The isolate from the suspected NEC neonate was found to be 100% resistant to all antibiotics except Aztreonam and Azithromycin, whereas of the 5 Escherichia coli isolated from controls, 100% were resistant to Linezolid and Vancomycin. Figures Fig 3.6c and 3.6d show the overall resistance to each antibiotic by *Escherichia coli* and *Klebsiella pneumoniae* isolates from suspected NEC and control neonates.

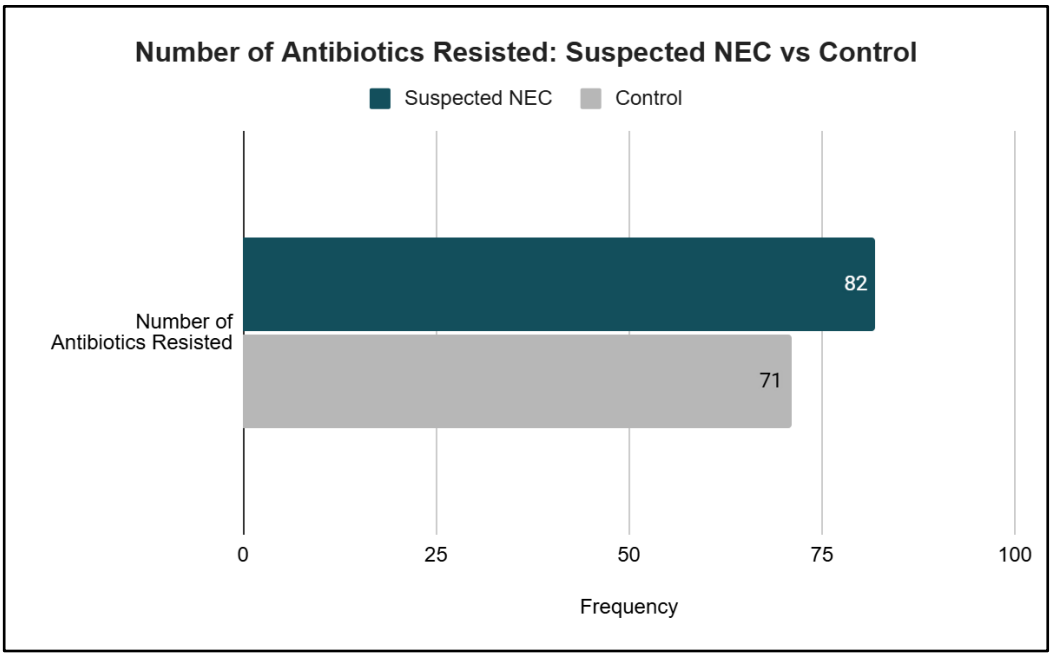

Fig 3.6a: Total number of antibiotics resisted by bacteria: Suspected NEC vs Control

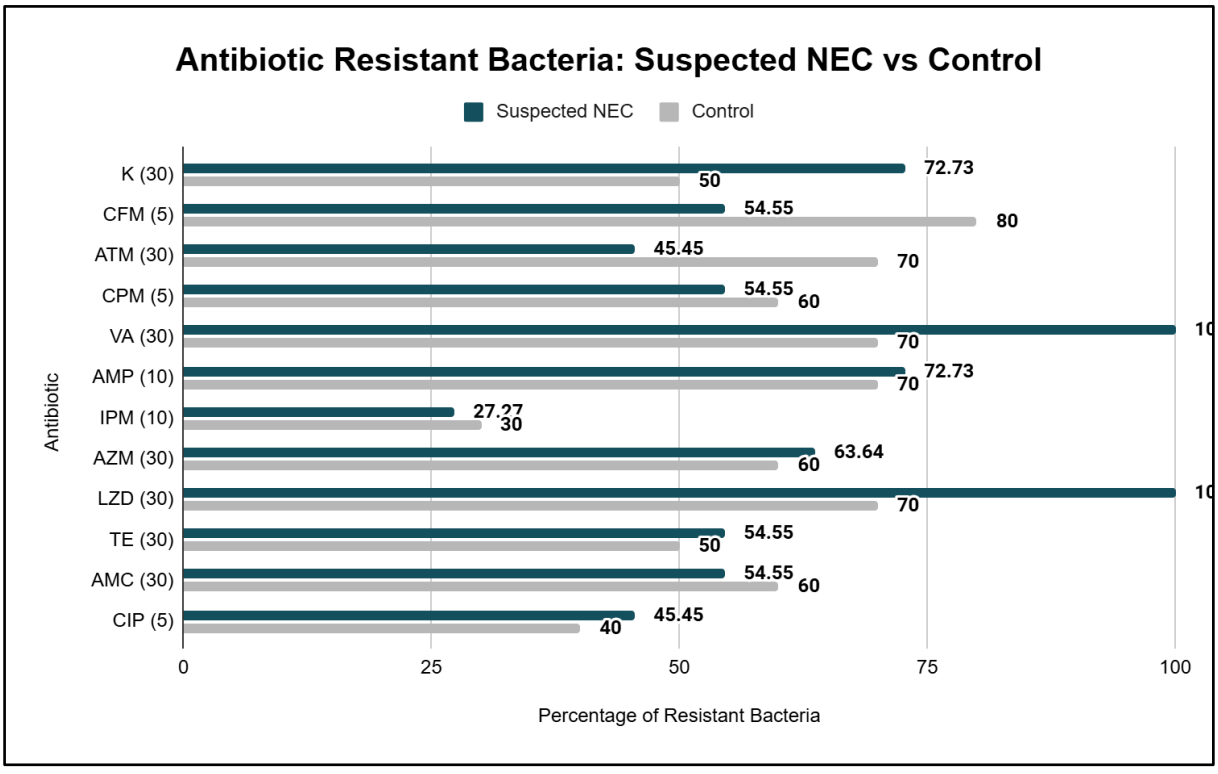

Fig 3.6b: Percentage of Resistance to each antibiotic: Suspected NEC vs Control

Table 3.6c: Percentage of each Bacteria: Case vs Control

Microorganism	Total Number	Case	Percentage of Case	Control	Percentage of Control
Escherichia coli	6	1	16.67	5	83.33
Klebsiella pneumoniae	11	6	54.55	5	45.45
Morganella morganii	1	1	100.00	0	0.00
Serratia marcescens	2	2	100.00	0	0.00
Enterobacter cloacae	1	1	100.00	0	0.00

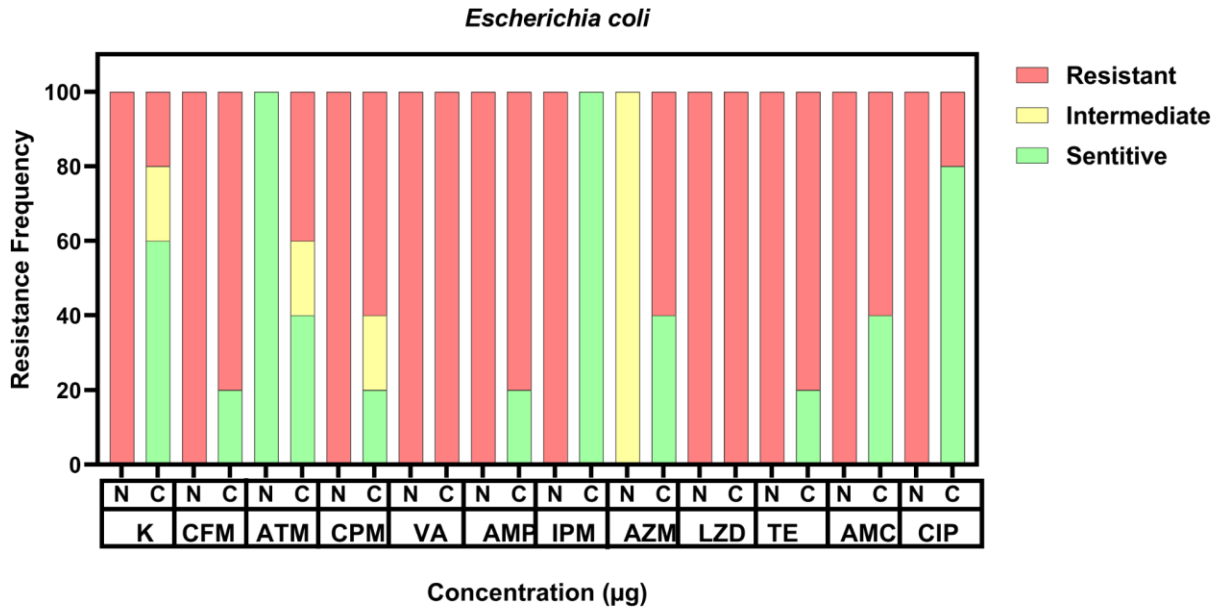

Fig 3.6c: **Resistance to each antibiotic by *Escherichia coli* isolates from suspected NEC cases and control neonates.** K: Kanamycin, CFM: Cefixime, ATM: Aztreonam, CPM: Cefepime, VA: Vancomycin, AMP: Ampicillin, IMP: Imipenem, AZM: Azithromycin, LZD: Linezolid, TE: Tetracycline, AMC: Amoxicillin, CIP: Ciprofloxacin

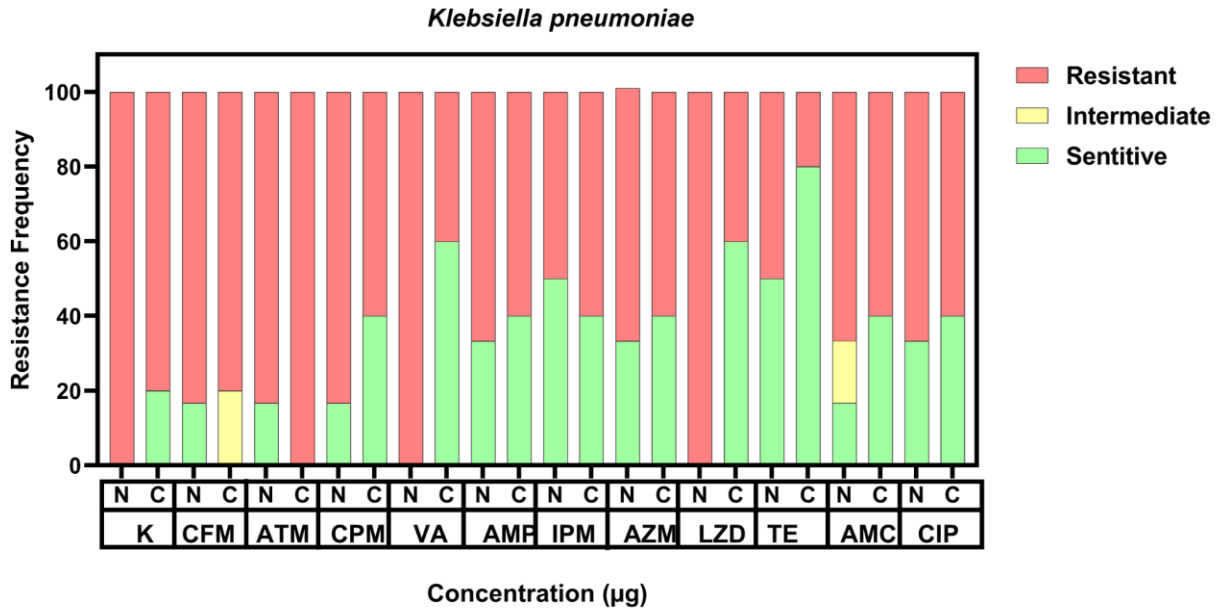

Fig 3.6d: Resistance to each antibiotic by *Klebsiella pneumoniae* isolates from suspected NEC cases and control neonates. K: Kanamycin, CFM: Cefixime, ATM: Aztreonam, CPM: Cefepime, VA: Vancomycin, AMP: Ampicillin, IMP: Imipenem, AZM: Azithromycin, LZD: Linezolid, TE: Tetracycline, AMC: Amoxicillin, CIP: Ciprofloxacin

Chapter 4: Discussion

4.1: Clinical and Demographic Data

Our findings have shown that despite NEC primarily affecting neonates that are designated in the weight class of VLBW (<1500g), (Meister et. al, 2019), 14 (46.67%) of suspected NEC neonates were above the VLBW weight class, with 4 (13.33%) neonates being above the WHO definition of Low Birth Weight (<2500g) (WHO, 2024). This may show that NEC is not just a disease that affects neonates of VLBW, but may also affect neonates that are in the LBW weight class or even at a healthy weight. Still, due to the small sample size, further investigation with a larger sample size is required.

Our findings have also shown that the majority of the suspected NEC neonates were born in one of the many categories that describe "preterm" (extremely preterm, very preterm, moderate to late preterm), which, as per the WHO guidelines, defines any neonate born with a gestational age of below 37 weeks (WHO, 2023). 25 (83.33%) of all suspected NEC neonates had a gestational age below 37 weeks. This aligns with the existing literature, highlighting the effect NEC has on preterm neonates.

The singular control that was later found to be a suspected NEC case provides invaluable insight into how the disease progresses within neonates. If we observe the CFU of the neonate before and after being diagnosed with suspected NEC, we find that the overall CFU for all other media such as Nutrient Agar, UTI and Blood Agar increase, whereas the CFU for MSA takes a sharp drop from 1500 to 50. However, the sample size is too insignificant to make any concluding statement, therefore further investigation with a larger sample size is required.

The average age of the mothers of NEC neonates was 24.85 years in comparison to the control group where the average age was higher, at 26.5 years. The ages are too similar to deduce any relationship between the age of mothers and the prevalence of NEC, and the median age for both groups are the same, coming to 24.5 years. Thus, it cannot be concluded that maternal age plays a significant role in the development of NEC.

Moving on to the clinical data, we begin to notice more differences between the suspected NEC group and the control group. No changes or significant changes were observed when comparing feeding difficulties between groups, which remained more or less the same with the suspected NEC group reporting 16 (53.3%) cases and the control group reporting 13 (44.8%) cases of feeding difficulties. Feeding methods within both groups remained relatively equal, with both groups seeing more formula feeding than breastfeeding, however the suspected NEC case group did report a higher percentage of neonates being formula fed in some capacity with 29 (96.67%) cases compared to the control group which saw 23 (79.31%) cases be formula fed in some capacity. A larger sample size may have allowed further investigation as many studies cite formula feeding as one of the major risk factors for developing NEC, citing the need for breast milk in the development of tolerance against bacterial microflora (Trinci et al., 2016, Alganabi et al., 2019, Hu et al., 2024).

The incidence of abdominal change was only observed in suspected NEC cases, with 16 (53.33%) neonates having experienced some type of abdominal change, with the control group reporting 0 cases of abdominal changes. These findings align with the existing literature where abdominal distention is widely regarded as one of the early symptoms for NEC, and is specifically mentioned in the Bell's Stage I classification (Alganabi et al., 2019; Meister et al., 2019, Hu et al., 2024). The incidence of vomiting was also seen more in the suspected NEC case group when compared to the control group, with 16 (53.33%) of neonates from the suspected NEC case group vomiting at least

once, compared to the control group which only had 2 (6.90%) cases. This aligns with the existing literature where vomiting has been associated with the development of NEC (Trinci et al., 2019; Neu, 2020). However, a larger sample size would have allowed the relationship to be further tested and provided a more robust conclusion.

Exploring the prescription of antibiotics between both groups, there is a clear difference as suspected NEC cases are prescribed with almost double the amount of antibiotics (122) when compared with the control group (62). This early exposure to antibiotics has also been linked with an increased likelihood of developing NEC, as the usage of such antibiotics leads to a decrease in the number of healthy microflora colonizing the gut in a process known as dysbiosis which increases the risk of pathogenic colonization (Alganabi et al., 2019; Meister et al., 2019; Sanchez et al., 2019; Neu, 2020). When taking a look at the most prescribed antibiotic for both groups, we see that for the suspected NEC group, the most prescribed antibiotic is Netilmicin (73.3%), Moxifloxacin (56.7%), followed by Meropenem and Vancomycin (50% each). In comparison, the most prescribed antibiotic for the control group was Imipenem (44.8%) followed by Gentamicin (41.4%) and then Moxifloxacin (31.0%). Netilmicin, Meropenem and Moxifloxacin are classified as broad-spectrum antibiotics, with Gentamicin being considered broad spectrum but is ineffective against streptococci and anaerobic bacteria, while Vancomycin is a narrow spectrum antibiotic that is used to treat staphylococcus infections (Rahman et al., 2020). The prescription of the broad spectrum antibiotics aligns with existing literature that says that the causative agent of NEC is not fully understood, so the employment of broad spectrum antibiotics hoping to quell the bacterial infection make sense (Meister et al., 2019; Neu, 2020;) Further studies show that in the event of sepsis, antibiotics such as Vancomycin and Gentamicin are prescribed (Duess et al., 2023; Hu et al., 2024). The high prescription of antibiotics in the suspected NEC case group aligns with existing literature that says that the prescription of these broad spectrum antibiotics causes dysbiosis which

ultimately leads to pathogenic colonization as mentioned earlier. Further investigation with a larger sample size and timeline is required to conclusively make a statement.

4.2: CFU Count of Samples

When exploring the overall CFU counts for both groups, it is evident that the overall CFU is lower in the suspected NEC group. If we compare the CFU counts for UTI agar, the suspected NEC group reports an average CFU of 5.31×10^7, which is almost 4 times less than the average CFU for the control group which is 2.07×10^8, comparing the data for MSA yields a similar result with the control group reporting an average CFU of 1.10×10^4, 3.5 times greater than the average for the suspected NEC group of 3.11×10^3, and even for Nutrient Agar it was the suspected NEC group reporting an average CFU of 5.25×10^7, more than 3 times less than the control group which reported an average CFU of 1.68×10^8. The difference wasn't as stark for Blood Agar, with the average CFU for the suspected NEC group being 1.15×10^8, which is a little less than the average reported in the control group of 1.24×10^8.

Exploring the existing literature gives very little data regarding the change in CFU for neonates affected with NEC, thus leaving only room for educated guesses. Given the almost double dosage of antibiotics received by the suspected NEC group, it would be reasonable to deduce that the high dosage of antibiotics had a significant impact on the overall microflora of the suspected NEC neonates, however, further investigation with a larger sample size is required to confirm this hypothesis.

4.3: Bacterial Species

The bacterial species identified through the use of selective and differential media proved to show similar results, but it is difficult to draw any conclusion from these due to the identities of the bacteria being suspected rather than confirmed. Further testing such as through MALDI-TOF or

VITEK, 2 options that were explored in this study are required to be able to draw valid conclusions from the results.

4.4 MALDI-TOF

The findings of the MALDI-TOF were interesting in the sense that it highlighted a difference in the microflora of both the control and the suspected NEC group. 5 different organisms that were absent in the control group were identified after carrying out MALDI-TOF on the suspected NEC group samples, these organisms include *Staphylococcus haemolyticus*, *Morganella morganii*, *Serratia marcescens*, *Candida tropicalis* and *Enterobacter cloacae*. Both *Enterobacter cloacae* and *Staphylococcus haemolyticus* have been suggested to have a relationship with the incidence of NEC, while one recent paper has shown the presence of *Serratia marcescens* in at least one neonate affected with NEC (Powell et al., 1980; GAŁĄZKA et al., 2021). The presence of *Candida tropicalis* is a fungus, with the Candida species being commonly associated with NEC patients, as it is responsible for 27% of all sepsis related deaths in those affected by NEC (Coggins et al., 2014). Most literature seems to agree that the most common candida species affecting NEC patients seems to *Candida albicans*, but the isolation of *Candida tropicalis* is interesting, and would require further investigation (Herran et al., 2010)

4.5 VITEK

The findings of our study indicated a significant presence of Gram-positive *Staphylococcus* species. However, discrepancies were observed between initial identifications and subsequent analyses. For instance, one control group sample initially suspected to contain *Staphylococcus aureus* was later identified as *Staphylococcus haemolyticus* through VITEK analysis.

Similarly, in the case group, a sample initially suspected to contain *Staphylococcus epidermidis* based on MSA agar growth was identified as *Kocuria kristinae* upon VITEK analysis. Another case group sample also suspected to contain *Staphylococcus epidermidis* was confirmed as *Streptococcus thoraltensis*.

Unexpectedly, some samples showed the growth of *Klebsiella pneumoniae* on MSA agar. This result was not anticipated and is likely due to contamination that occurred during sample processing.

4.6 Antibiotic susceptibility Test

The results of the antibiotic susceptibility test revealed a high prevalence of antibiotic-resistant bacteria in the gut microbiota of both groups. Determining whether or not a bacterium is resistant to antibiotics can be carried out through the use of the Multiple Antibiotic Resistance (MAR) Index. MAR index is calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to, with an MAR greater than 0.2 indicating high chance of resistance (Puspita et al., 2021). Across the 21 bacteria that were tested, 2 (9.5%) of them reported an MAR index of 1.0, which indicates that they are Multi Drug Resistant (MDR), with a further 14 (66.67%) reporting an MAR index of over 0.2, indicating high resistance to antibiotics. This is incredibly concerning given the fact that these are neonates that are barely 2 weeks old that already harbour MDR bacteria.

4.7 Strengths and Limitations:

This is the first type of study being conducted in Bangladesh to our knowledge. In our study, both the case and control groups were selected from the same hospital environment, specifically the NICU, which helped minimize potential biases, which is one of the strengths of our study.

However, the study has notable limitations. The small sample size of 60 participants may have contributed to the lack of statistical significance in the results and may not accurately reflect the true prevalence of NEC in Bangladesh. Additionally, samples were collected from a single tertiary hospital in Dhaka, limiting the ability to address local epidemiological factors that influence the development of NEC.

Another limitation is the exclusion of maternal history in some cases, which could have constrained our ability to explore potential associations between maternal risk factors and NEC. Lastly, the antibiogram patterns were analyzed for only ten infants, which significantly restricts the study's findings, as the small sample size does not adequately represent the antibiogram patterns of both study groups.

5. Appendix

5.1 Questionnaire:

Project Title: Exploring the Incidence of Suspected Necrotizing Enterocolitis (NEC) in Bangladesh: A Case-control Study

Patient ID:

Demographic Information

01.	Patient's Name		
02	Age		
03.	Gender	Male	Female
04.	Father's Name		
05.	Occupation		
06.	Mother's Name		
07.	Occupation		
08	Educational qualification of Parents	No education/Less than SSC / SSC or equivalent / HSC or equivalent / Bachelor or equivalent / Masters or equivalent / More than Masters	
09.	Monthly Income (BDT):	Monthly Expenditure (BDT):	

Clinical Information

01.	Weight (kg)		
02.	Height (cm)		
03.	Body Mass Index (BMI)		
04.	Types of feeding	Breast Milk	Enteral feeding

05.	Does the baby experience feeding difficulties in recent times?	Yes	No	
06.	Did you notice any changes in the baby's abdominal area, such as bloating or distention?	Yes	No	
07.	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
08.	Has the baby had any instances of fever?	Yes	No	If yes, how often
09.	Has the baby experienced any episodes of vomiting?	Yes	No	If yes, how often
10.	Has the baby received a blood transfusion after birth?	Yes	No	If yes, how many times

11.	Has the baby been administered with any enteral antibiotics after birth?	Yes	No	If yes, the Name of the antibiotics
12	Has the baby been given any probiotics, prebiotics, or both after birth?	Yes	No	
13.	Has the baby been given any additional growth factor (e.g., anti-cytokine or glucocorticoids)?	Yes	No	
14	Has the baby been given any intravenous hyperalimentation(A form of nutrition that is delivered into a vein)?	Yes	No	
15.	Has the baby undergone any surgical treatment?	Yes	No	If yes, When

Pregnancy history

01.	Types of delivery	Vaginal birth	C-section	VBAC

02.	Have you experienced any complications during delivery?	Yes	No	If yes, describe
03.	Have you experienced any mentionable complications during pregnancy?	Yes	No	If yes, describe
04.	Have you been exposed to any particular antibiotic during pregnancy?	Yes	No	If yes, the Name of the antibiotics:
05.	Which drug(s) was(were) suggested right after admission?			

5.2 Participant Information Consent Form

Project Title: Exploring the incidence and Risk Factors of Necrotizing Enterocolitis (NEC) in Bangladesh.

What is the purpose of the project?

To assess the status of necrotizing enterocolitis (NEC) in preterm neonates in Bangladesh.

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 associated disease. Participants will also provide their stool sample and blood sample (minimum volume required) for experimental analysis. Blood will be collected by the nurse of the respective hospitals.

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.

Will the participant be given the results of the research project?

Only aggregated non-identifiable data will be used to publish articles in scientific journals. If the participant would like to receive a copy of the publication, they can obtain one by contacting the principal investigator.

What will happen to clinical data and blood samples of the participant?

By signing the consent form, the parent of the participant consents to the study researcher collecting and using their baby's clinical data and blood sample for the research project.

Further information and who to contact

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 participant has understood that explanation

Name of the researcher: **Signature of the researcher:**

Date:

Table 5.3 Full List of Weight, Gestational Age of neonates and Mother Age: Suspected NEC vs Control

	Weight	Gestational Age	Mother Age
Suspected NEC	0.83	30+1	24
	1.02	34+5	28
	1.02	34+5	28
	1.05	35+6	20
	1.2	35+0	19
	1.2	35+0	19
	1.36	29+0	24
	1.36	31+4	31
	1.36	31+4	24
	1.4	32+5	24
	1.42	36+5	31
	1.46	30+2	-
	1.48	33+0	18
	1.49	36+1	20
	1.5	35+2	28
	1.5	35+2	28
	1.56	31+6	20

	1.6	36+4	25
	1.64	34+6	36
	1.88	34+6	26
	1.88	34+6	26
	1.9	36+4	25
	2.04	34+6	26
	2.2	37+6	23
	2.34	35+2	33
	2.4	40+0	18
	2.5	40+5	20
	2.6	36+1	28
	2.8	37+0	24
	3.1	40+3	-
Control	0.885	30+4	32
	1.01	29+0	38
	1.1	28	24
	1.1	32	28
	1.19	29	22
	1.19	30+0	25
	1.2	28	19

	1.26	34+2	26
	1.4	31+2	19
	1.5	34+2	32
	1.5	35+5	18
	1.6	33+0	32
	1.65	31+3	36
	1.79	36+0	22
	1.8	34+6	35
	1.9	36+0	-
	2.1	32+5	24
	2.1	35+1	20
	2.16	35+6	26
	2.16	36+2	19
	2.16	36+5	27
	2.29	35+3	26
	2.3	34+3	24
	2.3	34+5	28
	3.1	37+0	-
	3.39	37+6	35
	3.45	40+0	28

	3.5	40+0	35
	3.54	40+0	20

Table 5.4 Other Suspected Organisms as listed in Tables 3.3a and 3.3b

Sample Number	Suspected Organism
ADNEC 07	*Gamma Streptococcus*
ADNEC 11	*Acacinetobacter*
ADNEC 18	*Streptococcus pyogenes*
ADNEC 23	Bright Yellow ND on BAP
ADNEC 24	Colourless mucoid colony that caused hemolysis on BAP
ADNEC 28	Alpha hemolytic *streptococcus pneumonia, Pseudomonas aeruginosa*
ADNEC 30	*Streptococcus pyogenes*, UTI small yellow colony, UTI large white colony, BAP Yellow colony
ADNEC 32	UTI Mucoid Blue Centred, UTI Mucoid Bluish white centre, *Streptococcus pyogenes*
ADNEC 33	Yellow on BAP
ADNEC 36	BAP 10^-6 Light Brown

ADNEC 37	UTI 10^-3 light Pink, UTI 10^-5 Yellow N.D
ADNEC 41	BAP 10^-5 Colourless N.D
ADNEC 43	BAP 10^-5 Colorless N.D
ADNEC 49	BAP 10^-6 Brown Mucoid
ADNEC - 50	Yellow colony on BAP
ADNEC - 52	UTI 10^-3 Light Pink, BAP 10^-5 Yellow colony, BAP 10^-5 *Staphylococcus hemolyticus*
ADNEC - 53	*Streptococcus hemolyticus*, Colorless colony on BAP
ADNEC - 56	BAP colorless colony
ADNEC - 58	Colorless/Ash colony on BAP
ADNEC - 61	BAP 10^-6 Yellow colony, BAP 10^-6 Faded White colony on a chocolate background
ADNEC - 62	BAP 10^-6 Light Yelow Colony, BAP 10^-6 Dark Yellow Colony, BAP 10^-4 Light Pink Colony, *Streptococcus pyogenes*
ADNEC - 63	BAP 10^-6 Dark Yellow colony, BAP 10^-6 Faded White/Light Pink Colony, UTI 10^-5 Ash colony, UTI 10^-5 Greenish Mucoid
ADNEC - 64	*Streptococcus pyogenes*
ADNEC - 69	BAP 10^-6 Faded Yellow Colony
ADNEC - 70	UTI 10^-4 Colony with a deep blue centre and purple border

ADNEC - 71	BAP 10^-6 Ash mucoid colony, UTI 10^-3 Blue colony with a white border
ADNEC - 72	BAP 10^-5 Pink Small Colony
ADNEC - 73	BAP 10^-4 Bright Yellow colony, Light Yellow Colony, Greenish mucoid with lysis

Table 5.5 MALDI-TOF Results Summarized

Key for Table 5.5	
	Control
	Case

Case/Control	**Sample ID**	**Suspected Organism**	**Organism Matched**
Control	ADNEC - 02	*Streptococcus agalactiae*	*Escherichia coli*
Control	ADNEC - 02	*Listeria monocytogenes*	*Escherichia coli*
Control	ADNEC - 02	*Klebsiella pneumoniae*	-
Control	ADNEC - 02	Coagulase negative *Staphylococci*	*Klebsiella pneumoniae*
Control	ADNEC - 02	*Enterococcus faecalis*	-
Control	ADNEC - 02	*Saprophyticus*	-
Control	ADNEC - 05	*Staphylococcus epidermidis*	*Klebsiella pneumoniae*

Control	ADNEC - 05	*Staphylococcus aureus*	*Klebsiella pneumoniae*
Control	ADNEC - 05	*Streptococcus agalactiae*	*Klebsiella pneumoniae*
Control	ADNEC - 05	*Saprophyticus*	*Klebsiella pneumoniae*
Control	ADNEC - 07	*Gamma Streptococci*	*Escherichia coli*
Control	ADNEC - 07	*Saprophyticus*	*Klebsiella pneumoniae*
Control	ADNEC - 07	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Control	ADNEC - 07	*Listeria monocytogenes*	*Klebsiella pneumoniae*
Control	ADNEC - 10	*Klebsiella pneumoniae*	-
Control	ADNEC - 10	*Staphylococcus aureus*	*Klebsiella pneumoniae*
Control	ADNEC - 10	*Escherichia coli*	*Escherichia coli*
Control	ADNEC - 10	N.D	*Escherichia coli*
Control	ADNEC - 12	*Saprophyticus*	-
Control	ADNEC - 12	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Control	ADNEC - 12	*Escherichia coli*	*Escherichia coli*
Control	ADNEC - 12	*Staphylococcus epidermidis*	*Klebsiella pneumoniae*
Control	ADNEC - 12	N.D	*Klebsiella pneumoniae*

Case	ADNEC - 19	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Case	ADNEC - 19	*Enterococcus faecalis*	*Klebsiella pneumoniae*
Case	ADNEC - 19	*Staphylococcus epidermidis*	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Saprophyticus*	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Streptococcus agalactiae*	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Staphylococcus epidermidis*	*Candida tropicalis*
Case	ADNEC - 20	*Listeria monocytogenes*	*Klebsiella pneumoniae*
Case	ADNEC - 20	N.D	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Staphylococcus epidermidis*	*Klebsiella pneumoniae*
Case	ADNEC - 20	*Escherichia coli*	*Staphylococcus haemolyticus*
Case	ADNEC - 21	*Listeria monocytogenes*	*Morganella morganii*
Case	ADNEC - 21	*Escherichia coli*	*Escherichia coli*
Case	ADNEC - 21	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*

Case	ADNEC - 21	*Listeria monocytogenes*	*Morganella morganii*
Case	ADNEC - 21	*Streptococcus agalactiae*	*Klebsiella pneumoniae*
Case	ADNEC - 21	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Case	ADNEC - 21	*Saprophyticus*	*Morganella morganii*
Case	ADNEC - 29	*Streptococcus agalactiae*	-
Case	ADNEC - 29	*Staphylococcus aureus*	*Klebsiella pneumoniae*
Case	ADNEC - 29	*Saprophyticus*	*Serratia marcescens*
Case	ADNEC - 29	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Case	ADNEC - 29	*Enterococcus faecalis*	*Serratia marcescens*
Case	ADNEC - 31	*Streptococcus agalactiae*	*Serratia marcescens*
Case	ADNEC - 31	*Escherichia coli*	*Enterobacter cloacae*
Case	ADNEC - 31	*Enterococcus faecalis*	*Serratia marcescens*
Case	ADNEC - 31	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Case	ADNEC - 31	*Listeria monocytogenes*	*Klebsiella pneumoniae*
Case	ADNEC - 31	*Saprophyticus*	*Klebsiella pneumoniae*
Case	ADNEC - 31	*Staphylococcus aureus*	*Staphylococcus haemolyticus*

N.D: Not Determined

Table 5.6 AST Results: With Zones of Inhibition

Bacteria	Sample Number	Antibiotic	Zone 1 (mm)	Zone 2 (mm)	Zone 3 (mm)	Average Zone of Inhibition	AST
Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	ADNEC - 19	K (30)	0.0	0.0	0.0	0.0	Resistant
		CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	30.0	30.0	30.0	30.0	Sensitive
		AZM (30)	10.0	9.0	9.0	9.3	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	16.0	15.0	16.0	15.7	Intermediate
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
	ADNEC - 12	K (30)	15.0	15.0	20.0	16.7	Intermediate

Escherichia coli MB11464_1 CHB		CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	10.0	10.0	10.0	10.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	30.0	30.0	28.0	29.3	Sensitive
		AZM (30)	25.0	25.0	21.0	23.7	Sensitive
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	25.0	25.0	25.0	25.0	Sensitive
		AMC (30)	18.0	18.0	20.0	18.7	Sensitive
		CIP (30)	25.0	25.0	30.0	26.7	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	ADNEC - 12	CFM (5)	5.0	1.0	2.0	2.7	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	16.0	19.0	17.0	17.3	Sensitive
		VA (30)	28.0	28.0	29.0	28.3	Sensitive
		AMP (10)	35.0	35.0	30.0	33.3	Sensitive
		IPM (10)	43.0	42.0	38.0	41.0	Sensitive
		AZM (30)	30.0	30.0	30.0	30.0	Sensitive
		LZD (30)	35.0	35.0	30.0	33.3	Sensitive
		TE (30)	30.0	30.0	25.0	28.3	Sensitive
		AMC (30)	35.0	35.0	32.0	34.0	Sensitive
		CIP (30)	23.0	22.0	25.0	23.3	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	ADNEC - 05	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	11.0	11.0	10.0	10.7	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	9.0	9.0	8.0	8.7	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	11.0	10.0	10.0	10.3	Resistant
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	20.0	20.0	20.0	20.0	Sensitive
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	21.0	20.0	20.0	20.3	Sensitive

Escherichia coli DSM 682	ADNEC - 02	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	18.0	18.0	19.0	18.3	Sensitive
		CPM (5)	14.0	14.0	13.0	13.7	Intermediate
		VA (30)	8.0	9.0	8.0	8.3	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	28.0	28.0	29.0	28.3	Sensitive
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	8.0	8.0	7.0	7.7	Resistant
		AMC (30)	9.0	10.0	9.0	9.3	Resistant
		CIP (30)	30.0	30.0	30.0	30.0	Sensitive
		K (30)	18.0	18.0	19.0	18.3	Sensitive

Escherichia coli MB11464_1 CHB	ADNEC - 02	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	17.0	16.0	16.0	16.3	Intermediate
		CPM (5)	12.0	12.0	14.0	12.7	Resistant
		VA (30)	10.0	8.0	9.0	9.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	28.0	27.0	23.0	26.0	Sensitive
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	10.0	10.0	10.0	10.0	Resistant
		CIP (30)	29.0	28.0	30.0	29.0	Sensitive
		K (30)	28.0	28.0	29.0	28.3	Sensitive

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	ADNEC - 05	CFM (5)	16.0	16.0	17.0	16.3	Intermediate
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	19.0	19.0	21.0	19.7	Sensitive
		VA (30)	28.0	28.0	27.0	27.7	Sensitive
		AMP (10)	40.0	41.0	40.0	40.3	Sensitive
		IPM (10)	46.0	46.0	47.0	46.3	Sensitive
		AZM (30)	30.0	20.0	26.0	25.3	Sensitive
		LZD (30)	39.0	40.0	39.0	39.3	Sensitive
		TE (30)	38.0	37.0	38.0	37.7	Sensitive
		AMC (30)	43.0	41.0	40.0	41.3	Sensitive
		CIP (30)	31.0	32.0	34.0	32.3	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB	ADNEC - 20	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	9.0	9.0	11.0	9.7	Resistant
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	20.0	20.0	19.0	19.7	Sensitive
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp ozaenae DSM 16358T DSM	ADNEC - 20	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	0.0	0.0	0.0	0.0	Resistant
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	25.0	25.0	23.0	24.3	Sensitive

Serratia marcescens ssp marcescens DSM 30121T DSM	ADNEC - 31	CFM (5)	28.0	30.0	30.0	29.3	Sensitive
		ATM (30)	35.0	35.0	32.0	34.0	Sensitive
		CPM (5)	30.0	30.0	28.0	29.3	Sensitive
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	20.0	20.0	20.0	20.0	Sensitive
		IPM (10)	27.0	27.0	29.0	27.7	Sensitive
		AZM (30)	12.0	15.0	16.0	14.3	Intermediate
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	10.0	10.0	10.0	10.0	Resistant
		AMC (30)	12.0	15.0	13.0	13.3	Intermediate
		CIP (30)	30.0	30.0	32.0	30.7	Sensitive
		K (30)	20.0	20.0	20.0	20.0	Sensitive

Enterobacter cloacae ssp cloacae ESBL2036 JUG	ADNEC - 31	CFM (5)	30.0	30.0	30.0	30.0	Sensitive
		ATM (30)	30.0	35.0	35.0	33.3	Sensitive
		CPM (5)	30.0	30.0	30.0	30.0	Sensitive
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	23.0	23.0	21.0	22.3	Sensitive
		IPM (10)	30.0	30.0	30.0	30.0	Sensitive
		AZM (30)	10.0	10.0	10.0	10.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	25.0	25.0	25.0	25.0	Sensitive
		AMC (30)	25.0	25.0	25.0	25.0	Sensitive
		CIP (30)	23.0	25.0	25.0	24.3	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Escherichia coli DH5alpha BRL	ADNEC - 21	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	27.0	27.0	28.0	27.3	Sensitive
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	8.0	8.0	8.0	8.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	17.0	17.0	12.0	15.3	Intermediate
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	8.0	8.0	8.0	8.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB	ADNEC - 31	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	10.0	10.0	10.0	10.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	12.0	12.0	13.0	12.3	Resistant
		AZM (30)	22.0	28.0	22.0	24.0	Sensitive
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	24.0	22.0	21.0	22.3	Sensitive
		AMC (30)	8.0	9.0	9.0	8.7	Resistant
		CIP (30)	22.0	20.0	20.0	20.7	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	ADNEC - 21	CFM (5)	24.0	22.0	22.0	22.7	Sensitive
		ATM (30)	30.0	28.0	29.0	29.0	Sensitive
		CPM (5)	21.0	21.0	22.0	21.3	Sensitive
		VA (30)	8.0	9.0	9.0	8.7	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	31.0	31.0	31.0	31.0	Sensitive
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	16.0	19.0	18.0	17.7	Sensitive
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	0.0	0.0	0.0	0.0	Resistant

Morganella morganii ssp morganii	ADNEC - 21	CFM (5)	28.0	27.0	30.0	28.3	Sensitive
		ATM (30)	34.0	38.0	35.0	35.7	Sensitive
		CPM (5)	30.0	31.0	29.0	30.0	Sensitive
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	21.0	21.0	22.0	21.3	Sensitive
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	7.0	7.0	6.0	6.7	Resistant
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	28.0	30.0	26.0	28.0	Sensitive
		K (30)	28.0	28.0	27.0	27.7	Sensitive

Serratia marcescens 13103_1 CHB	ADNEC - 29	CFM (5)	30.0	30.0	29.0	29.7	Sensitive
		ATM (30)	31.0	35.0	31.0	32.3	Sensitive
		CPM (5)	30.0	30.0	27.0	29.0	Sensitive
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	16.0	17.0	18.0	17.0	Intermediate
		IPM (10)	27.0	30.0	27.0	28.0	Sensitive
		AZM (30)	19.0	19.0	20.0	19.3	Sensitive
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	19.0	19.0	17.0	18.3	Sensitive
		AMC (30)	16.0	18.0	19.0	17.7	Sensitive
		CIP (30)	31.0	32.0	30.0	31.0	Sensitive
		K (30)	8.0	8.0	8.0	8.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB 2	ADNEC - 29	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	10.0	10.0	10.0	10.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	17.0	18.0	17.0	17.3	Sensitive
		AZM (30)	23.0	22.0	21.0	22.0	Sensitive
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	23.0	22.0	21.0	22.0	Sensitive
		AMC (30)	8.0	7.0	8.0	7.7	Resistant
		CIP (30)	24.0	24.0	25.0	24.3	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB 2	ADNEC - 10	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	0.0	0.0	0.0	0.0	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	0.0	0.0	0.0	0.0	Resistant
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	20.0	18.0	18.0	18.7	Sensitive

Escherichia coli MB11464_1 CHB	ADNEC - 10	CFM (5)	20.0	23.0	20.0	21.0	Sensitive
		ATM (30)	30.0	30.0	30.0	30.0	Sensitive
		CPM (5)	30.0	30.0	29.0	29.7	Sensitive
		VA (30)	10.0	12.0	12.0	11.3	Resistant
		AMP (10)	20.0	20.0	20.0	20.0	Sensitive
		IPM (10)	31.0	30.0	30.0	30.3	Sensitive
		AZM (30)	29.0	21.0	25.0	25.0	Sensitive
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	9.0	8.0	10.0	9.0	Resistant
		AMC (30)	22.0	23.0	24.0	23.0	Sensitive
		CIP (30)	29.0	28.0	25.0	27.3	Sensitive
		K (30)	0.0	0.0	0.0	0.0	Resistant

Escherichia coli *DSM 682*	ADNEC - 07	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	11.0	12.0	12.0	11.7	Resistant
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	17.0	18.0	17.0	17.3	Sensitive
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	0.0	0.0	0.0	0.0	Resistant
		TE (30)	0.0	0.0	0.0	0.0	Resistant
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant
		K (30)	0.0	0.0	0.0	0.0	Resistant

Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB	ADNEC - 07	CFM (5)	0.0	0.0	0.0	0.0	Resistant
		ATM (30)	0.0	0.0	0.0	0.0	Resistant
		CPM (5)	0.0	0.0	0.0	0.0	Resistant
		VA (30)	25.0	24.0	25.0	24.7	Sensitive
		AMP (10)	0.0	0.0	0.0	0.0	Resistant
		IPM (10)	0.0	0.0	0.0	0.0	Resistant
		AZM (30)	0.0	0.0	0.0	0.0	Resistant
		LZD (30)	32.0	30.0	33.0	31.7	Sensitive
		TE (30)	29.0	29.0	27.0	28.3	Sensitive
		AMC (30)	0.0	0.0	0.0	0.0	Resistant
		CIP (30)	0.0	0.0	0.0	0.0	Resistant

Table 5.7 AST Results: Resistance to Antibiotics

Key for Table 5.7	
	Control
	Case
	MDR

Case/Control	Sample	Bacteria	Resistance to Antibiotic (Number)	Antibiotics that are resisted by Bacteria
Control	ADNEC - 02	*Escherichia coli DSM 682*	7	CFM (5) VA (30)

				AMP (10) AZM (30) LZD (30) TE (30) AMC (30)
	ADNEC - 02	*Escherichia coli MB11464_1 CHB*	8	CFM (5) CPM (5) VA (30) AMP (10) AZM (30) LZD (30) TE (30) AMC (30)
	ADNEC - 05	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB (BAP)*	11	CPM (5) VA (30) AMP (10) AZM (30) LZD (30) TE (30) AMC (30) K (30) CFM (5) ATM (30)

				CPM (5) VA (30) AMP (10) IPM (10) AZM (30) LZD (30) AMC (30) CIP (5)
	ADNEC - 05	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB (UTI)*	1	ATM (30)
	ADNEC - 07	*Escherichia coli DSM 682*	11	ATM (30) K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) AZM (30) LZD (30) TE (30) AMC (30) CIP (5)

	ADNEC - 07	*Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB*	9	K (30) CFM (5) ATM (30) CPM (5) AMP (10) IPM (10) AZM (30) AMC (30) CIP (5)
	ADNEC - 10	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB 2*	12	K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) IPM (10) AZM (30) LZD (30) TE (30) AMC (30) CIP (5)
	ADNEC - 10	*Escherichia coli MB11464_1 CHB*	3	VA (30) LZD (30)

				TE (30)
	ADNEC - 12	*Escherichia coli MB11464_1 CHB*	6	CFM (5) ATM (30) CPM (5) VA (30) AMP (10) LZD (30) VA (30) LZD (30) TE (30)
	ADNEC - 12	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB*	3	K (30) CFM (5) ATM (30)
Case	ADNEC - 19	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB*	10	K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) AZM (30) LZD (30) TE (30) CIP (5)

	ADNEC - 20	*Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB*	11	K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) IPM (10) AZM (30) LZD (30) AMC (30) CIP (5)
	ADNEC - 20	*Klebsiella pneumoniae ssp ozaenae DSM 16358T DSM*	12	K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) IPM (10) AZM (30) LZD (30) TE (30) AMC (30) CIP (5)

	ADNEC - 21	*Escherichia coli DH5alpha BRL*	10	K (30) CFM (5) CPM (5) VA (30) AMP (10) AZM (30) LZD (30) TE (30) AMC (30) CIP (5)
	ADNEC - 21	*Morganella morganii ssp morganii*	7	K (30) VA (30) AMP (10) AZM (30) LZD (30) TE (30) AMC (30)
	ADNEC - 21	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB 2*	7	K (30) VA (30) AMP (10) AZM (30) LZD (30) TE (30)

				CIP (5)
	ADNEC - 29	*Serratia marcescens 13103_1 CHB*	2	VA (30) LZD (30)
	ADNEC - 29	*Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB 2*	8	K (30) CFM (5) ATM (30) CPM (5) VA (30) AMP (10) LZD (30) AMC (30)
	ADNEC - 31	*Serratia marcescens ssp marcescens DSM 30121T DSM*	3	VA (30) LZD (30) TE (30)
	ADNEC - 31	*Enterobacter cloacae ssp cloacae ESBL2036 JUG*	3	VA (30) AZM (30) LZD (30)
	ADNEC - 31	*Klebsiella pneumoniae ssp pneumoniae 9295 1 CHB*	9	K (30) CFM (5) ATM (30) CPM (5)

				VA (30) AMP (10) IPM (10) LZD (30) AMC (30)

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