ISOLATION, IDENTIFICATION, AND ANTIBIOTIC SENSITIVITY PATTERN OF ORAL MICROFLORA FROM CHILDREN WITH NEURODEVELOPMENTAL DISORDERS IN COMPARISON TO TYPICALLY DEVELOPING CHILDREN

By

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A thesis submitted to the Programme of Biotechnology in the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of B.Sc. in Biotechnology

Department of Mathematics and Natural Sciences

Brac University

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Declaration

It is hereby declared that

1. The thesis submitted is my/our original work while completing the 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 that has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I/We have acknowledged all main sources of help.

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Approval

The thesis/project titled "Isolation, Identification and Antibiotic Sensitivity pattern of Oral Microflora from Children with Neurodevelopmental Disorders in Comparison to Typically Developing Children**"**

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Dedicated to

My grandmother, ***Mrs. Bilkis Begum***, whose unwavering belief in me and boundless dreams have been a constant source of inspiration.

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Abstract

Neurodevelopmental disorders impact brain function and development, leading to difficulties in social, cognitive, and emotional areas. A study by the Neuro-Developmental Disability Protection Trust, 2020 reported that 3 million people in Bangladesh are diagnosed with autism spectrum disorder and other Neurodevelopmental disorders. Additionally, a 2013 pilot study found that 7.1% of Bangladesh's population has some form of neurodevelopmental disorder. Recent research suggests a potential link between the oral microbiome and autism, with individuals with NDs at higher risk of oral health issues that can affect their oral microbiome. In this study, the oral microbiota of children with neurodevelopmental disorders was compared to that of neurotypical children, along with an analysis of antibiotic susceptibility patterns in the identified microorganisms. This study revealed notable differences between the isolates from the control and sample groups. Oral swabs were taken from 20 children with NDs, including those diagnosed with Autism, ADHD, Asperger's syndrome, Cerebral Palsy, and both mild and high-functioning autism. For comparison, 40 samples were taken from neurotypical children. Although the control group had twice as many samples, the number of isolates was not proportional, with 78 isolates found in the control group and 54 in the sample group, suggesting a higher number of bacterial isolates in the sample group. In the control group, 27% of the isolates were gram-positive and 73% were gram-negative, while in the sample group, 61% were gram-positive and 39% were gram-negative. Notably, the sample group had more than twice as many *staphylococcus* species compared to the control group, with 52%, compared to 19% in the control group. This indicates a greater vulnerability to bacterial colonization in children with NDs. The antibiotic susceptibility analysis further highlighted significant challenges, as children with NDs showed higher resistance to

several key antibiotics compared to the control group. Although some similarities were found in resistance patterns for Gram-positive isolates, there were significant differences for Vancomycin, Doxycycline, and Levofloxacin, with the sample group showing much higher resistance rates with 87.5% for Vancomycin, 62.5% for Doxycycline, and 68.8% for Levofloxacin, compared to 45.5%, 31.8%, and 45.5%, in the control group. The situation was even more concerning for Gram-negative isolates, where the sample group demonstrated much higher resistance across multiple antibiotics. For example, resistance to Levofloxacin and Doxycycline was 90.5% in the sample group, while the control group showed much lower resistance rates of 18.6% for both. This pattern was consistent across other antibiotics, with the ND group showing resistance rates of 95.3% for Azithromycin, 95.2% for Ampicillin, and 57.2% for Gentamicin, compared to 56%, 61%, and 15.2% in the control group. The data gained from this study depicts the distinct difference in bacterial presence between neurotypical individuals and children with neurodevelopmental disorders as well as their antibiotic susceptibility pattern. PCR was performed to confirm the isolates of *staphylococcus aureus* and *staphylococcus species*. The PCR test confirmed the presence of *Staphylococcus aureus* in 15% of the controls and in 22.5% of the samples. Moreover, other *Staphylococcus species* were confirmed to be present in 45% of the controls and in 95% of the Samples.

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Chapter 1: Introduction and Literature Review

1.1 Neurodevelopmental Disorder

Neurodevelopmental disorders or NDs are those that affect a person's brain function and development, causing them to have complications in social, cognitive, and emotional areas. The most common NDs are ASD or autism spectrum disorder and ADHD or attention-deficit/hyperactivity disorder. (May et al., 2019). NDs commonly develop in childhood, however can persist into adulthood or the whole life of a person. (Hansen et al., 2018)

Various conditions are included in the Neurodevelopmental disorder category such as learning disabilities, intellectual disability, and cerebral palsy. Males are more likely to have a neurodevelopmental disorder probably due to their genetic and biological risk factors. However, the symptoms of such disorders are the same in both males and females. Neurodevelopmental disorders are so common that globally 15-20% of the children are affected by it. In the us, 17.8% of the children aged between 3 to 17 are affected by some sort of Neurodevelopmental disorder which is rising even in middle-aged and older adults, suggesting that the number will rise to 700,000 by 2030.

The prevalence and impact of neurodevelopmental disorders as well as their increasing rates should be strictly addressed to develop effective interventions and support systems through continued research.

1.2 Causes of Neurodevelopmental Disorder

The exact causes of neurodevelopmental disorders are yet to be discovered. However, it is believed that biological and environmental factors play a key role in developing NDs. While genetic or

hormonal reasons can be the root of ND development, environmental risk factors such as stress, illness, or trauma can accelerate the process. (May et al., 2019)

Mutations, deletions, or duplications of specific genes responsible for brain development and function are the crucial genetic causes of neurodevelopmental disorders. Genetic alterations can interrupt the normal process of neuronal development, differentiation, and connectivity thus leading to abnormalities in the brain. For example, a mutation in the MECP2 gene can cause Rett syndrome, on the contrary, deletions in the 22q11.2 chromosome can lead to DiGeorge syndrome. (Sauer et al., 2019)

Correspondingly, the study that focuses on how behavioral and environmental factors can alter DNA and gene function is called Epigenetics. Studies show that early childhood experiences such as trauma, neglect, or toxic environment can be risk factors for developing NDs. (Hansen et al., 2018) Moreover, prenatal stress, nutrition, and exposure to toxins that cause DNA methylation or histone modification are also very vital in developing NDs as they can affect gene expression. (Petersen et al., 2020).

1.3 Neurodevelopmental Disorders in Bangladesh

The research on Neurodevelopmental disorders in Bangladesh is highly inadequate, however, some progress has taken place recently. The government of Bangladesh has started to address these issues with the help of urban and rural communities and NGOs. (Hossain et al., 2020)

According to a study by Neuro-Developmental Disability Protection Trust in 2020, currently, there are 3 million people in Bangladesh who are diagnosed with ASD and other NDs. Another pilot study done in 2013 found that 7.1% of the total population of Bangladesh has some type of

neurodevelopmental disorder. (Hossain et al., 2017) Autism is a rapidly growing ND in Bangladesh which was confirmed by Mullick and Rabbani, finding through their study that the rates of autism per 1000 children increased from 0.2 to 0.84% between the years 2005 and 2009. Another study held in 2016 claims that 19% of all recorded neurological disabilities were autism. (Ministry of Social Welfare, 2016)

The Neuro-Developmental Disability Protection Trust has taken various initiatives, such as setting up service centers across the country which will provide a wide range of services, including early intervention, different types of therapy, counseling, and training for parents and caregivers with the help of specialists like physiotherapists, occupational therapists, speech and language therapists, clinical psychologists, and medical doctors (Neuro-Developmental Disability Protection Trust, 2020). Despite these initiatives, the research and outputs regarding neurodevelopmental disorders are very limited. Comprehensive research is needed to understand and support people suffering from these disorders.

1.4 Types and Symptoms of Neurodevelopmental Disorder

There are many types of neurodevelopmental disorders (NDDs). Here are some of the most common ones:

- Autism spectrum disorder (ASD)
- Attention-deficit/Hyperactivity Disorder (ADHD)
- Asperger’s Syndrome
- Cerebral Palsy
- Communication disorders
- Intellectual disorders
- Neurodevelopmental Motor Disorders etc.

1.4.1 Autism spectrum disorder (ASD)

Autism spectrum disorder or ASD refers to conditions that are characterized by challenges one faces with social interaction, communication, and repetitive behaviors. ASD causes difficulties in transitioning from one activity to another, focusing on details, and having uncommon sensory reactions. 1 in 100 children is diagnosed with autism, globally. (WHO, 2022)

Autism is usually recognized at an early age, in the first two years of one's life, it however can be diagnosed at any age. It is diagnosed by evaluating a person's behavior. People who have ASD often suffer from sleep difficulties and self-injuries. The intellectual abilities however can vary from profound impairment to superior intelligence. (NIMH, 2018)

Some of the common signs of ASD are:

- Limited eye contact
- Difficulty in social interaction and communication
- Repetitive behaviors or intense interests
- Sensitivity towards light, sound, touch, and other sensory elements

Some of the strengths observed in people with ASD are:

- Strong memory skills
- Excellent visual and auditory learning abilities
- Talents in specific areas. (NIMH, 2018)

1.4.2 Attention-Deficit/Hyperactivity Disorder (ADHD)

ADHD is one of the most common neurodevelopmental disorders that usually affect children and can continue into adulthood. Children with ADHD exhibit abnormal levels of hyperactivity, impulsivity, and inattention, thus disrupting their daily lives. (Harpin, 2005) If the proper treatment is not provided, people with ADHD might end up developing poor self-esteem and social difficulties. (Beaton et al., 2022)

The number of people diagnosed with ADHD has increased significantly over the last two decades. According to a study done in 1997, 6.3% children of aged 5 to 17 had ADHD. Later on, in 2021, another study showed that the number has increased almost double, to a 10.8%. ADHD has also been found to be more common in males than females with a percentage of 14.1% and 7.5% respectively.

There are three types of ADHD which are:

1. Predominantly inattentive presentation
2. Predominantly hyperactive/impulsive presentation
3. Combined presentation

1.4.3 Asperger's Syndrome

Asperger’s syndrome falls under the category of autism spectrum which is known as a high-functioning form of ASD, affecting the behavior and social interactions of a person. (Autism Society, 2023). People with Asperger's syndrome often have difficulties with social interactions, they stick to routines have narrow interests, and might also show repetitive behaviors like hand flapping (WebMD, 2023).

People with Asperger’s syndrome show various symptoms such as:

- Not understanding social cues and rules
- Speaking unusually, for example, using formal language or a monotone
- Difficulty making friends and understanding others' thoughts
- Sensitivity to light and sound

1.4.4 Cerebral Palsy

Cerebral Palsy or CP occurs as a result of damage caused to the developing brain during pregnancy, birth, or shortly after birth. (CDC, 2023). CP can affect the movement, muscle control, coordination, tone, reflexes, posture as well as balance of the person affected. The muscle weakness may worsen with wear and tear over time due to the condition. 1 in 323 children are affected by CP in the U.S. with a concerning number of 18 million people living with the condition globally. (CDC, 2023) Although the exact cause is unknown in most cases, two significant risk factors have been found to be prematurity and stroke. (NIH, 2023)

People with CP may also experience trouble swallowing, eye muscle imbalance, and reduced joint motion along with other symptoms. (Mayo Clinic, 2023) On top of that, people may also have additional impairments with a shocking number of 75% having chronic pain, 33% being unable to walk and or having hip displacement, 25% having verbal communication difficulties, 50% having intellectual impairment along with lesser common impairments such as impaired vision, autism, impaired hearing and many more. (Cerebral Palsy Alliance, 2023)

1.5 Neurodevelopmental disorders and oral microbiota

Recent studies show that there might be a connection between the oral microbiome and autism spectrum disorders. People with neurodevelopmental disorders have a higher risk of oral health problems, which can alter the oral microbiome (Como et al., 2021; Qiao et al., 2018). Studies have found significant differences in oral microbiome composition between autistic and non-autistic children, linking these differences to autism-related behaviors (Ragusa et al., 2020; Qiao et al., 2018; Hicks et al., 2018; Abdulhaq et al., 2021; Kong et al., 2019).

A study exhibited that by transferring oral microbiota from children with ASD and typically developing children into microbiota-depleted mice, the ASD microbiota induced ASD-like behaviors in the mice, with a different structure than those receiving TD microbiota. Additionally, the prefrontal cortex of mice receiving ASD microbiota showed altered gene expression related to serotonin, neuroactive ligand-receptor interactions, and TGF-β signaling. Specific oral microbiota, such as *Bacteroidetes, Porphyromonas,* and *Tannerella,* were linked to these changes. This study highlighted the oral microbiome to brain connections in ASD development, providing a novel strategy to understand ASD etiology. (Liu et al., 2020)

Moreover, multiple studies suggest dysbiosis impacts autism severity and GI tract issues through the gut-brain axis by emphasizing the role of the gut microbiome in autism. (Cryan et al., 2019; Mangiola et al., 2016; Adams et al., 2011; Mayer et al., 2019) Furthermore, evidence suggests that there are bidirectional interactions between the oral microbiome and brain processes, potentially influencing conditions like autism and Alzheimer's (Bowland & Weyrich, 2022; Qiao et al., 2018). Also, the research project by Bowland and Weyrich, suggests that through an oral-microbiota-

brain axis (OMBA), oral microbiota may influence brain processes and NPD outcomes (Bowland & Weyrich, 2022). Last but not least, research was done, comparing the oral microbiome of 80autistic children with 40 typically developing children which revealed that certain bacteria such as *Solobacterium, Stomatobaculum,* and *Ruminococcus* etc. are more abundant in autistic children which relates to social difficulties, repetitive behaviors and anxiety. However, more studies need to be conducted to confirm the connection between the brain and the oral microbiome.

1.6 Treatment for Neurodevelopmental Disorders

Although there is no cure for neurodevelopmental disorders, research has determined there are methods that help with managing and coping with symptoms. Some of these are:

Behavioral Therapy: Behavioral therapies such as Applied Behavioral Analysis (ABA), cognitive-behavioral therapy (CBT), speech therapy, and developmental therapy have been shown to be beneficial for NDD symptoms. ABA is particularly effective for children with NDDs like ASD and ADHD while CBT and other behavioral therapies are often used for adults with NDDs. (Smith et al., 2020).

Transcranial Magnetic Stimulation: Repetitive transcranial magnetic stimulation is a therapy that uses magnetic fields to stimulate nerve cells. It is commonly used for depression but has shown potential benefits for NDDs. (Davis et al., 2019).

Medication: Medication can be prescribed for both children and adults with NDDs. However, medication is not always the first choice. Experts often recommend trying behavioral therapies and parenting training before resorting to medication for conditions like ADHD and ASD (American Academy of Pediatrics, 2019).

Conventional and Alternative Medicine: Healthy lifestyle habits such as good nutrition, regular physical exercise, and engaging in recreational activities are important for managing NDD symptoms. CAM treatments are more commonly used by adults with NDDs, with around 36-38% of adults engaging in such therapies. However, the use of CAM by children with NDDs varies widely, with estimates ranging from 2% to 55% (National Center for Complementary and Integrative Health, 2020).

1.7 Oral microbiota

The oral microbiome, comprising bacteria, archaea, fungi, viruses, and protozoa, is intricately linked to oral health (Gomez & Nelson, 2017). The diversity of oral microorganisms increases in children after initial colonization. A balance between the host and these microorganisms is maintained through complex interactions which however if disrupted, can lead to oral diseases. (Lamont et al., 2018). The oral microbiota is also connected to systemic diseases, including head and neck cancer, pancreatic cancer, colorectal cancer, rheumatoid arthritis, hypertension, Alzheimer's disease, etc. (Liu et al., 2019; Pessoa et al., 2019). Traditionally, studies focused on bacteria due to limitations in culturing techniques, but advancements in sequencing and molecular methods have expanded our understanding to include fungi, viruses, and a unique class of bacteria known as the candidate phyla radiation (CPR) group (Baker et al., 2017).

1.7.1 *Staphylococcus*

Bacteria from the genus *Staphylococcus*, primarily *Staphylococcus aureus*, a common pathogen responsible for a range of infections in humans. These infections appear on the skin as pus-filled lumps, abscesses, boils, or scabs, and can lead to more severe conditions that affect various body

parts such as the skin, breasts, digestive system, bones, lungs, heart, and bloodstream. Symptoms vary from localized pain and redness to septicemia and toxic shock syndrome. Diagnosis of *staphylococcus* can be done through clinical examination, Gram stain tests, bacterial cultures, and sometimes imaging or blood tests. Treatment of *Staphylococcus* includes antibiotics, while prevention includes good hygiene, wound care, and avoiding contact with contaminated sources. Hospitalized patients, those with chronic conditions or weakened immune systems, children, and individuals who inject drugs or are breastfeeding are at the highest risk for such infections. (Cleveland Clinic, n.d.)

1.7.2 *Klebsiella*

The genus *Klebsiella,* of the family *Enterobacteriaceae,* includes nonmotile, rod-shaped, gram-negative bacteria characterized by a polysaccharide capsule that protects host defenses (Cleveland Clinic, n.d.) The polysaccharide capsule enhances virulence by resisting host defenses. *Klebsiella pneumoniae* is commonly found in the intestines and is typically harmless. However, if they enter other parts of the body, they pose significant risks, especially in individuals already suffering from weak immune systems. *Klebsiella* can develop antibiotic resistance, thus causing serious conditions such as pneumonia, wound infections, and bloodstream infections (Cleveland Clinic, n.d.)

1.7.3 *Shigella*

Shigella is a Gram-negative, facultatively anaerobic, non-spore-forming, nonmotile, and rod-shaped genus of bacteria that is genetically closely related to *Escherichia* (CDC, 2023). *Shigella* infection is called shigellosis which is an intestinal infection with the primary symptom often being

bloody diarrhea. Children under the age of 5 are particularly susceptible to shigella infection, although individuals of any age can contract the infection. (Mayo Clinic, 2023).

Shigella is highly contagious and spreads through contact with small amounts of bacteria from the stool of an infected person. *Shigella* bacteria can also be transmitted through contaminated food or by drinking or swimming in unsafe water (CDC, 2023).

1.7.5 *Enterobacter* species

Enterobacter species are a part of the Enterobacteriaceae family which are Gram-negative, rod-shaped and facultatively anaerobic bacteria. They are commonly found in soil, water, sewage, as well as in the intestines of humans and animals. The most clinically significant species are *Enterobacter cloacae* and *Enterobacter aerogenes* which are also known as opportunistic pathogens as they primarily cause infections in individuals with weakened immune systems. They can lead to a variety of hospital-acquired infections. Enterobacter species show a rapid development in terms of antibiotic resistance, thus making the treatment challenging. (Davin-Regli & Pages, 2015)

1.7.6 *Coagulase-negative staphylococci (CoNS)*

Coagulase-negative staphylococci or CoNS are a diverse group within the genus *Staphylococcus* that lack the coagulase enzyme found in *Staphylococcus aureus.* They include species like *Staphylococcus epidermidis, Staphylococcus haemolyticus, and Staphylococcus lugdunensis,* mostly found on human skin and mucous membranes (Kloos and Bannerman, 2002).

CoNS were once considered benign; however, they have recently appeared as opportunistic pathogens, particularly in healthcare settings where they colonize medical devices like catheters

and prosthetic implants, forming biofilms that resist immune defenses. They cause infections such as urinary tract infections, bloodstream infections, and surgical site infections, creating challenges due to their multidrug resistance (Kloos and Bannerman, 2002).

1.8 Use of Antibiotics and antibiotic resistance

Antimicrobials include antibiotics, antivirals, antifungals etc. which prevent and treat infections in humans, plants, and animals. When bacteria, viruses, or other microbes evolve and no longer respond to antimicrobials, it is called antimicrobial resistance or AMR. This resistance makes the antibiotics ineffective and makes it impossible to treat diseases. Antibiotics are powerful medications that either stop bacteria from reproducing or destroy them, thus treating certain infections and saving lives when used properly. (Medical News Today) There are two working methods of antibiotics, bactericidal which kills bacteria by disrupting cell walls while bacteriostatic antibiotics stop bacterial multiplication.

The increasing amount of resistance to antimicrobial medications impacts not only the individual but the overall health sector, pushing the world to return to a pre-antibiotic era. Antibiotic resistance can occur due to human activities such as misuse or overuse of antibiotics for treatment, not considering the proper uses of it. Antibiotic resistance can hinder the ability to treat common infections and even make performing critical procedures difficult.

Antibiotic resistance occurs in countries of all income levels. The significant causes identified include access to clean water, sanitation and hygiene, inadequate disease prevention, poor access to quality and affordable vaccines, lack of awareness, etc. (WHO) The 2022 Global Antimicrobial

Resistance and Use Surveillance System (GLASS) report emphasizes the gravity of antibiotic resistance, revealing alarmingly high resistance rates among common bacterial pathogens. (GLASS) According to GLASS, the resistance rate median in 76 countries is 42% for third-generation cephalosporin-resistant E. coli and 35% for methicillin-resistant staphylococcus aureus. Moreover, a 2020 report expressed that one in every five cases of UTI caused by E. coli showed reduced susceptibility to antibiotics such as ampicillin, co-trimoxazole and fluoroquinolones thus making common infections complicated to treat.

1.9 Antibiotic Resistance in Bangladesh

According to WHO, southeast Asia is at the highest risk for antimicrobial resistance. Although the countries in this region have policies for antimicrobial use in food and poultry, the regulatory enforcement is still non-functional thus making the implementation of the policies ineffective. This issue further causes the ineffectiveness of environmental contamination policies regarding uses of antimicrobial residues.

One significant issue in Bangladesh is that antimicrobial drugs are found over the counter, making them much more available. On top of that, the unqualified providers in the informal health sector and unethical marketing practices by pharmaceutical companies make the situation worse.

According to many studies, 66% of children and 44% of adults are prescribed antimicrobials in Bangladesh. ceftriaxone, ciprofloxacin, azithromycin and other higher-generation antimicrobials are prescribed frequently. To make things worse, multiple antimicrobials are prescribed simultaneously. Due to a lack of facilities, often these antibiotics are prescribed without laboratory tests. According to research, around 50% of antibiotic resistance is caused as patients stop taking medications when symptoms improve, ignoring the prescribed dosage. Moreover, self-treatment

is also popular which is influenced by prior experience, advice from traditional healers etc. with 36% treating diarrhea, and food poisoning, 28% treating colds and fevers, and 13% treating presumed infections.

Studies show that resistance against common antibiotics is very common in Bangladesh. For instance, 64% *Salmonella typhi* and 84% *E. coli* isolated from urine samples showed multi-drug resistance. Antimicrobial residues were also found in poultry meat and eggs, with a concerning rate of 50-60% in different samples. Poultry manure, on the other hand, used in aquaculture contains antimicrobial residues that contaminate water and vegetable. Street foods in different cities also contained bacterial samples that showed multidrug resistance, thus adding concerning components to the issue. Collectively all these factors put Bangladesh at a high risk of antimicrobial resistance.

1.11 Objectives

1. To identify and compare the Oral microbiota among children with neurodevelopmental disorders and neurotypical children.

2. To observe and compare the susceptibility pattern against common antibiotics in the retrieved microorganisms.

Chapter 2: Materials and Methods

2.1 Study Place

The laboratory work for this research was conducted in the Biotechnology and Microbiology lab of the Mathematics and Natural Sciences Department, Brac University.

2.2 Study Duration

The duration of this research work at the lab was held from March 2023 to March 2024.

2.3 Study Population

A total of 60 samples were collected for this study, of which 20 were of children with neurodevelopmental disorders, and the other 40 were collected from healthy children. The samples were collected from a school for special needs children, “Positive thinking” and a normal school, “Shahajpath High School”. All of the samples were collected from children aged between 6 to 17.

2.4 Sample Collection

2.4.1 Swab Sample Collection

Samples were collected using a sterile swab. Sterile swab sticks were labeled and after sanitizing the hands, the sealing cap was removed and kept aside then the swab was taken out carefully, making sure that it didn’t touch any other surface. After that, the swab was gently placed into the oral cavity of the selected person, and using a rolling motion, the swab tip was moistened by the saliva samples. The swab was placed on both sides of the oral cavity as well as underneath the tongue.

2.4.2 Bacterial Collection

The swab samples were immediately taken to the laboratory where sterile physiological saline tubes were already prepared for dilution. The sample swab was placed in a test tube and vortexed which further followed serial dilution. Following that, the diluted samples were spread into Nutrient agar media, MacConkey agar media, Xylose Lysine Deoxycholate agar media, and Mannitol Salt agar media for identifying different colonies for further examination. Once the colonies formed after 24 hours of incubation, they were streaked onto the desired media for isolated colonies.

2.4.3 Data Collection

While collecting the samples, the name, age, and grade of study was taken of the children as well as the diagnosis of the children with neurodevelopmental disorders.

Age: All of the children were aged between 6-17.

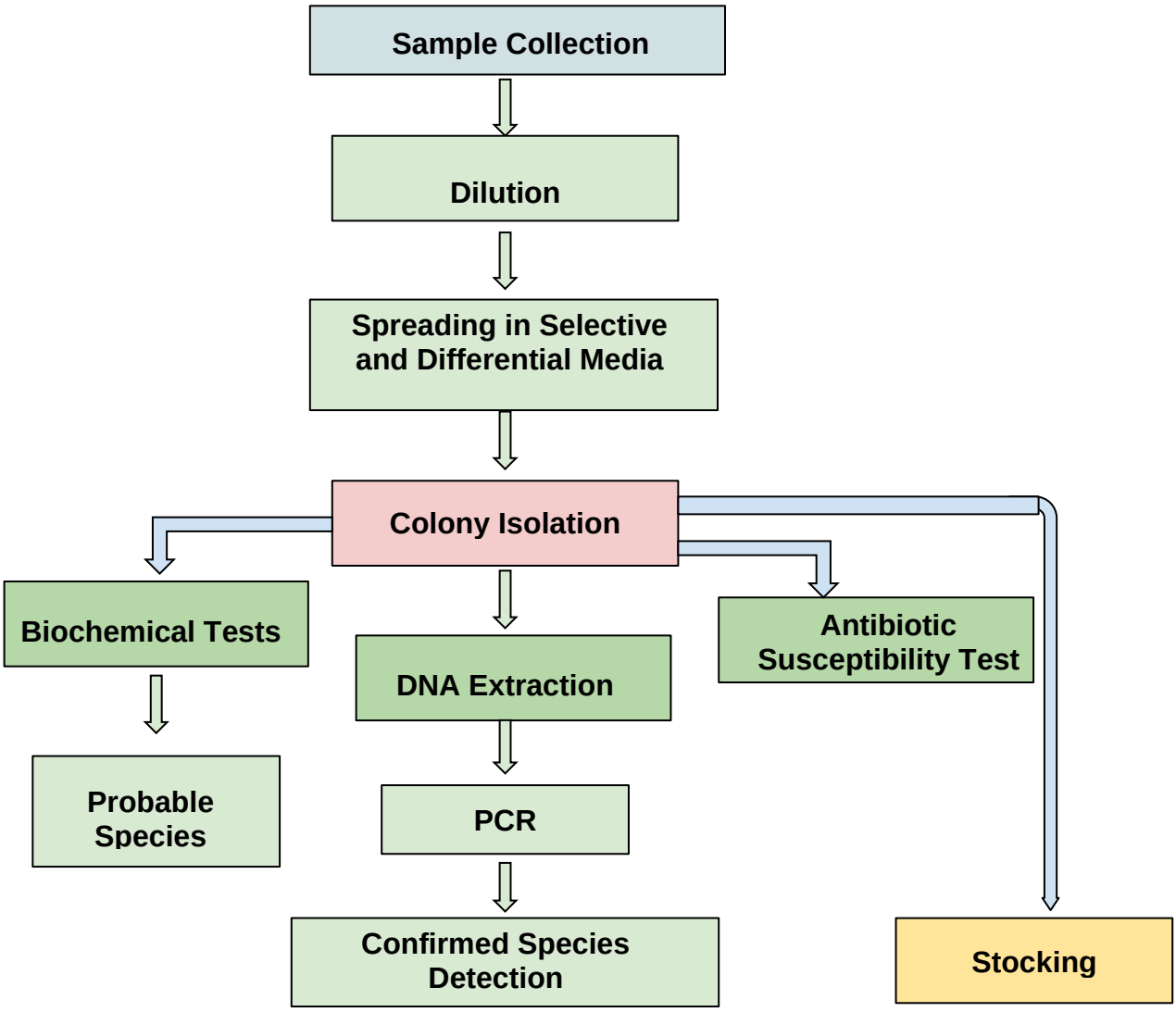
Grade of study: The children were students of grades 1 to 9.

Diagnosis of the diseased group: There were children with the diagnosis of **Autism, ADHD, Asperger's syndrome, Cerebral Palsy, and Mild and High functioning autism**.

2.5 Laboratory Equipment Used

- Autoclave machine
- Laminar Airflow Cabinet
- Incubator
- Microscope
- Vortex Machine
- PCR machine
- Gel electrophoresis machine
- Micropipette
- Laboratory water distillation apparatus
- pH meter
- Electric balance
- Glasswares: Petri-dish, test tube, vial, conical flask, Duran Bottle etc.
- Plasticwares: Microcentrifuge tube, PCR tube, Pipette tips etc.

2.6 Experimental Workflow



2.7 Culture Media for Bacterial Isolation

Different culture media were prepared for the identification of the bacterial isolates.

2.7.1 Nutrient Agar

Nutrient agar (NA) is the most common and major culture medium that allows the growth of non-fastidious microorganisms. Microorganisms are grown in NA for isolation before further testing. NA is an ideal medium as it allows a variety of types of bacteria and fungi to grow and contains nutrients for more prolonged survival of cultures. This media contains 0.5% peptone which is an enzymatic digest of animal protein, which works as organic nitrogen for the growth of bacteria. It also contains 0.3% beef or yeast extract which aids in bacterial growth. Moreover, the 0.5% salt or sodium chloride present in the medium maintains a salt concentration that is similar to the microorganism's cytoplasm. Furthermore, Distilled water acts as a mode through which nutrients are transported. Lastly, 0.5% agar acts as the solidifying agent. The media is prepared by adding 28g of Nutrient Agar powder to 1 litre of distilled water and upon heating and dissolving the powder, the media is autoclaved and plated for further use.

2.7.2 Mannitol Salt Agar

Mannitol Salt agar or MSA is a selective and differential medium that allows the isolation and identification of Staphylococcus aureus from clinical and non-clinical specimens. Its selective and differential characteristic causes it to inhibit the growth of gram-negative microorganisms and help to differentiate mannitol fermenters from non-fermenters. This media contains a Pancreatic digest of casein, Peptic digest of Animal Tissue and Beef extract in 0.5%, 0.5% and 0.1% concentrations to work as the nutrient source for the microorganisms to grow. It also contains 7.5% salt or sodium

chloride which partially or completely inhibits organisms other than staphylococci as well as supplying electrolytes for transport and osmotic balance. Moreover, Mannitol is added in 1% concentration which is the key element that is added as the source of fermentable carbohydrates which once fermented, can cause acid production and with the help of phenol red (0.0025%) indicator leads to the detection and differentiation of staphylococci species. Finally, 1.5% agar is added to solidify the medium. The media is prepared by adding 111.025g of Mannitol Salt Agar powder to 1 liter of distilled water and upon heating and dissolving the powder, the media is autoclaved and plated for further use. The different organisms and their visible characteristics are shown in the table below:

Microorganisms	**Visible characteristics**
Staphylococcus Aureus	Yellow colonies with yellow zones
Coagulase negative staphylococci	Colorless or red colonies with red zones
Gram Negative bacteria	No growth

Table 2.1: Mannitol Salt Agar

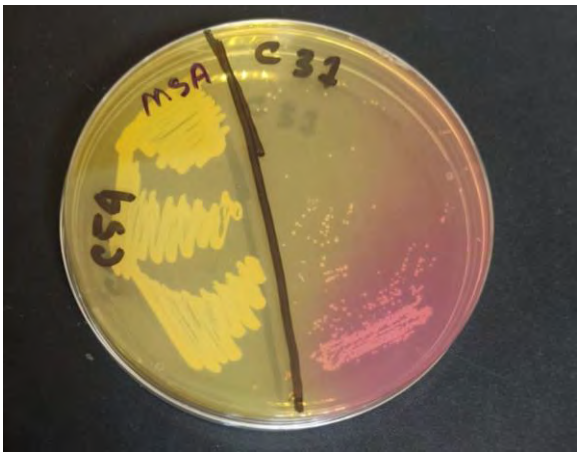


Figure2.1: Mannitol Salt agar (Mannitol fermenting and non-fermenting microorganism)

2.7.3 MacConkey Agar

MacConkey agar was developed in the 20th century by Alfred Theodore MacConkey which was also the first solid differential media to be made. It is a selective and differential media for isolating and differentiating non-fastidious gram-negative rods, mostly for the family Enterobacteriaceae and the genus Pseudomonas. It differentiates between lactose fermenting and non-fermenting gram-negative bacteria. 1.7% Pancreatic digest of gelatin and 0.3% peptones of meat and casein are added to the media as the nutrient source for bacterial growth. In this media, 1% lactose monohydrate works as the fermentable carbohydrate source. The selectivity of this media is ensured by 0.15% bile salt and 0.0001% crystal violet which inhibits the growth of most gram-positive bacteria. Moreover, 0.5% salt or sodium chloride is added to maintain the osmotic balance of the media. In addition, the pH indicator Neutral Red determines the change in pH of the medium upon lactose monohydrate fermentation. Lastly, 1.35% agar is added to solidify the media. The media is prepared by adding 49.53g of Mannitol Salt Agar powder to 1 liter of distilled water and upon heating and dissolving the powder, the media is autoclaved and plated for further use. The different organisms that grow on this media and their characteristics are given below:

Microorganisms	**Visible characteristics**
Klebsiella pneumoniae	Pale pink, mucoid colonies
Aerobacter aerogenes	Mucoid, pink colonies
Enterococcus species	Red, round colonies
Pseudomonas aeruginosa	Green-brown fluorescent colonies
Staphylococcus species	Pale pink, opaque colonies

Table 2.2: MacConkey Agar media

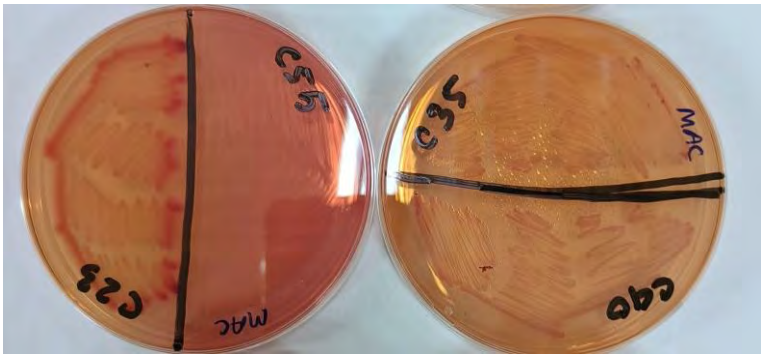


Figure2.2: MacConkey Agar plate

2.7.4 Xylose Lysine Deoxycholate Agar

Xylose Lysine Deoxycholate is a medium that is both selective and differential for the isolation of Gram-negative enteric pathogens from fecal specimens and clinical material. Yeast extract (0.3%) works as the nutrient and vitamin source of the medium. Sodium deoxycholate (0.5%) is present in the media to give it a selective property, thus inhibiting the growth of gram-positive microorganisms. The XLD media is highly suitable for differentiating species of *Shigella* and *Salmonella*. The Xylose ().37%) present in the medium makes it possible to differentiate *Shigella* species as *Shigella* cannot ferment xylose while all other enterics can. Meanwhile, the Lysine (0.5%) differentiates *Salmonella* species from nonpathogens as the presence of lysine prevents *Salmonella* from causing rapid fermentation of xylose and therefore distinguishing the species. Once xylose is completely exhausted by the fermentation of *Salmonella* species, it starts using lysin through lysine decarboxylase, causing an alkaline pH, making it similar to *Shigella*. Lactose (0.75%) and sucrose (0.75%) produce acid in excess to prevent similar reversion by lysine-positive coliforms. The indicator added to the medium to detect the degradation of xylose, lactose and sucrose to acid is Phenol Red (0.008%) which turns yellow upon acid production. Sodium thiosulfate (0.68%) and Ferric ammonium citrate (0.08%) create a Hydrogen Sulfide indicator

system in the media that aids in visualizing hydrogen sulfide production, marked by colonies with black centers. Finally, agar (0.15%) is added as the solidifying agent and the media is heated and poured to a petri dish for further use. The different organisms that grow on this media and their characteristics are given below:

Microorganisms	**Visible characteristics**
Salmonella Typhi	Red colonies, black centers
Salmonella Choleraesuis	Red colonies
Shigella Species	Red colonies
Proteus Vulgaris	Yellow colonies
Gram-positive Bacteria	No Growth

Table2.3: Xylose Lysine Deoxycholate agar

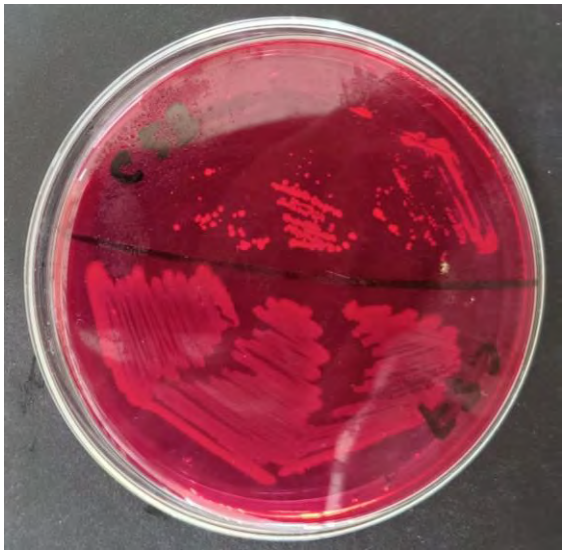


Figure2.3: Xylose Lysine Deoxycholate Agar plate

2.7.5 HiChrome UTI Agar

The Hichrome UTI agar media is usually used to detect urinary tract pathogens. Its hichrome activity allows for more distinct isolation of microorganisms. The media allows to identification of different microorganisms depending on the different colors of colonies produced by the specific enzymes of the genus or species upon interaction with two chromatic substrates present in the media. These chromogenic substrates get cleaved by enzymes such as ß-glucosidase of Enterococci And ß-D-galactosidase of *E.coli* to give Blue and pink colonies, whereas coliforms show a purple color as they cleave both of the substrates. Species such as *Proteus* and *Morganella* show tryptophsn deaminase activity and thus give a brown color colony in the media. The colonies and their color are stated in the table below:

Genus/species	**Visible characteristics**
Enterococci	Blue
E. Coli	Pink
Coliform	Purple
Proteus	Brown
Morganella	Brown

Table2.4: HiChrome UTI agar

2.8 Biochemical Tests

Biochemical tests are the traditional method of identifying microorganisms through their phenotypic reaction and biochemical activities towards different biochemical compounds. Different microorganisms can utilize different biomolecules and produce useful organic compounds, detecting those compounds is the main focus of the biochemical tests. Microorganisms can be classified into groups or even species depending on these tests. Biochemical tests are comparatively inexpensive and easier to conduct thus making them highly essential in the lab.

2.8.1 Gram Staining

Gram staining was developed in 1884 by Hans Christian Gram which is used to differentiate bacteria into gram-positives and gram-negatives based on their cell wall structure. This technique is essential for the study of bacterial morphology and classification. Crystal violet is used as the primary stain and safranine is used as a counterstain. The primary dye Crystal violet dissociates in water into CV+ and Cl– ions. These ions can penetrate bacterial cell walls and form CV-I complexes inside bacterial cells upon the addition of Gram's Iodine. When a decolorizing solution such as ethanol or acetone is added, Gram-negative bacteria lose the complexes due to their thin peptidoglycan layer of the cell wall. On the other hand, Gram-positive bacteria retain these complexes in their cell, appearing violet even after the wash. Safranin is added as the counterstain to detect Gram-Negative bacteria which turn pink due to negative components, while dehydrated Gram-Positive bacteria remain violet due to trapped CV-I complexes.

Examples of Gram stain results:

Gram-positive bacteria:

Gram-positive cocci– *Staphylococcus, Streptococcus and Enterococcus* species, etc.

Gram-positive bacilli: *Bacillus, Clostridium, Lactobacillus, Streptomyces, Listeria* species etc.

Gram-Negative bacteria:

Gram negative cocci– *Neisseria, Moraxella, Acinetobacter* species etc.

Gram negative bacilli- *E. coli, Klebsiella, Salmonella, Shigella, Pseudomonas* species etc.

2.8.2 Triple Sugar Iron Test

The triple sugar iron test determines the ability of microorganisms' to ferment carbohydrates and produce hydrogen sulfide by using the triple sugar iron agar. The agar contains 0.1% glucose and 1% of lactose and sucrose each. The test differentiates microorganisms based on their fermentation of these sugars and hydrogen sulfide production. The phenol red indicator present in the media indicates a pH change upon sugar fermentation and production of acid, turning the media from red to yellow if acid is present. On the other hand, oxidative decarboxylation of peptone in aerobic conditions produces alkaline products, thus causing the indicator to turn the media red. The glucose concentration is kept at one-tenth of the concentration of lactose or sucrose to specifically identify microorganisms that only ferment glucose. Since the concentration is very little, it produces acid in a small amount as well. This minimal amount of acid rapidly oxidizes in the slant of the tube, making it red or yellow depending on the pH. However, the butt of the tube remains in an acidic condition, keeping it yellow and thus indicating glucose fermentation.

Moreover, for the detection of hydrogen sulfide production, the media contains sodium thiosulfate and ferrous ammonium which react with hydrogen sulfide and form black ferrous sulfide in the tube's butt, giving a visible confirmation.

Figure 2.4: TSI test

Procedure:

1. TSI agar media was prepared and poured into test tubes, keeping in a slant until it solidified.
2. The top of an isolated colony was touched with a straight needle.
3. Inoculation was done by stabbing through the center of the TSI slant and then streaking through the slant surface.
4. The cap was loosely placed and incubated at 37°C for 24hrs.
5. Result was observed.

Probable Results are given in the table below:

Result	Observation	Interpretation
Alkaline slant, Acid butt	Red slant, yellow butt	Dextrose fermentation only
Acid slant, Acid butt	Yellow slant, yellow butt	Fermentation of dextrose, lactose and/or sucrose.
Alkaline slant, Alkaline butt	Red slant, red butt	Absence of carbohydrate fermentation
Hydrogen Sulfide production	Blackening of the medium	Presence of H_2
Gas production	Bubbles or cracks in the agar	Gas formation of CO_2 and H_2

Table 2.5: Triple sugar Iron Test

2.8.3 Citrate Utilization Test

The citrate test is often also referred to as Simmon's citrate test as it uses Simmon's citrate agar. The Simmons Citrate agar is specifically designed to determine the ability of an organism to utilize citrate as the sole carbon source. This test aids in identifying the Enterobacteriaceae family of microorganisms based on their metabolic capabilities. The sole carbon source of this medium is citrate while inorganic ammonium salts work as the nitrogen source. Organisms that are capable of utilizing citrate can produce enzymes like citrase which breaks down citrate into oxaloacetate and acetate. The Oxaloacetate then further gets metabolized into pyruvate and carbon dioxide. This releases carbon dioxide, thus increasing the pH of the medium through the conversion of ammonium salts into ammonia or sodium carbonate. The reaction increases the pH of the media and turns the bromothymol blue indicator from green to blue above pH 7.6, indicating a positive test result.

$$\textbf{Citrate} \rightarrow \textbf{Oxaloacetic acid} \rightarrow \textbf{Pyruvic acid} + CO_2$$

$$\textbf{Excess of sodium from sodium citrate} + CO_2 + H_2O \rightarrow Na_2CO_3$$

Bacteria that yield a positive reaction: *Salmonella, Edwardsiella, Citrobacter, Klebsiella* etc.

Bacteria that yield a negative reaction: *Escherichia, Shigella, Morganella,* and *Yersinia*.

Proteus shows variable response to citrate test.

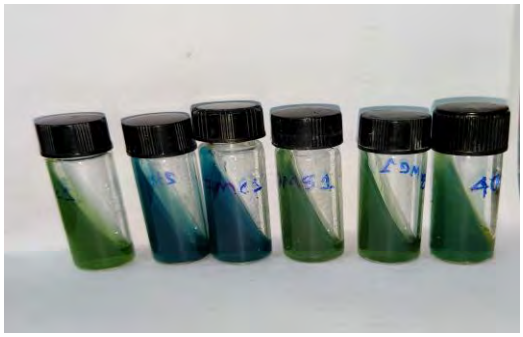


Figure 2.5: Citrate Utilization test

Procedure:

1. The Simmons citrate agar media was prepared.

2. The media was distributed into vials, creating a slant.

3. An isolated colony from a fresh culture was taken with a sterile needle

4. The media was stabbed and streaked through the slant using the needle.

5. The vials were incubated at 37°C for up to 72 hours.

6. Result was observed.

The result interpretation is as follows:

Observation	**Interpretation**
Media turns Blue	Positive result
Media remains green	Negative result

Table 2.6: Citrate Test

2.8.4 Catalase

The Catalase test is one of the fundamental biochemical assays that helps to identify organisms based on their ability to produce the catalase enzyme as part of aerobic metabolism. The catalase enzyme breaks down hydrogen peroxide into water and molecular oxygen. The chemical reaction of the catalase enzyme is as follows: $H_2O_2 \rightarrow H_2O + O_2$

Catalase reaction is highly essential to neutralize the hydrogen peroxide produced through aerobic respiration as it can be toxic for organisms living in oxygen-rich environments. Catalase activity can be visualized by the rapid formation of oxygen bubbles upon the addition of hydrogen peroxide

to the bacterial culture, thus showing a positive result. On the other hand, where no bubbles are formed, it indicates a catalase-negative result. This test gives insights into the metabolic characteristics of bacteria and aids in their classification. The results and examples of the test results are given below.

Observation	**Result**	**Example**
Bubble formation	Positive	*Staphylococci, Micrococci*
No bubble	Negative	*Streptococci*

Table 2.7: Catalase test

Procedure:

1. A microscope slide is taken and a single colony of the desired organism is taken from a 24 hour fresh culture using a sterile inoculating loop and placed on the slide.
2. A few drops of 3% Hydrogen peroxide (H_2O_2) is poured onto the organism on the microscope slide by using a dropper.
3. The formation of bubbles is observed.

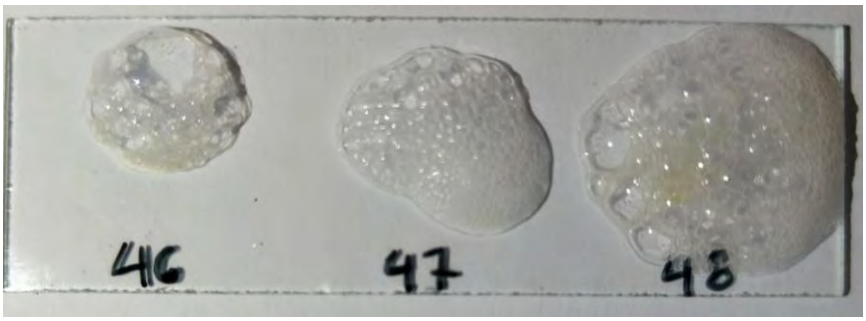


Figure 2.6: Catalase test

2.8.5 Oxidase

Oxidase test is used to detect whether a cytochrome oxidase enzyme is present in the bacteria or not. This oxidase enzyme is an important element of the bacterial respiratory chain which works in electron transportation. A redox dye, tetramethyl-p-phenylenediamine (TMPD) is used in this test. TMPD is usually colorless but turns deep purple when it comes in contact with the cytochrome oxidase enzyme. Organisms that contain cytochrome, have intracellular oxidase enzymes that oxidize cytochrome c. This oxidation process transfers electrons from donor compounds such as NADH to an acceptor such as oxygen. The TMPD reagent acts as an artificial electron acceptor, therefore cytochrome oxidase-containing bacteria when exposed to this reagent, forms indophenol blue, a colored compound that can be visibly seen as blue or purple. On the other hand, bacteria not containing cytochrome oxidase cannot oxide TMPD and do not show a color change. Different reagents can be used for this test such as Kovacs Oxidase Reagent, Gordon and McLeod's Reagent, and Gaby and Hadley or indophenol oxidase Reagent. These reagents contain tetramethyl-p-phenylenediamine dihydrochloride or related compounds in different concentrations dissolved in water or ethanol.

The observation and test results are discussed in the table below:

Observation	Result	Example
Color change to Blue/Purple	Positive	*Pseudomonas, Neisseria, Vibrio*
No change in color/colorless	Negative	*Enterobacteriaceae*

Table 2.8: Oxidase test

Procedure

1. A strip of filter paper is taken and an isolated colony is taken from a 24 hours fresh culture using a sterile loop or swab and smeared onto the filter paper.
2. The filter paper is soaked with a little freshly made 1% solution of the reagent.
3. Result is observed.

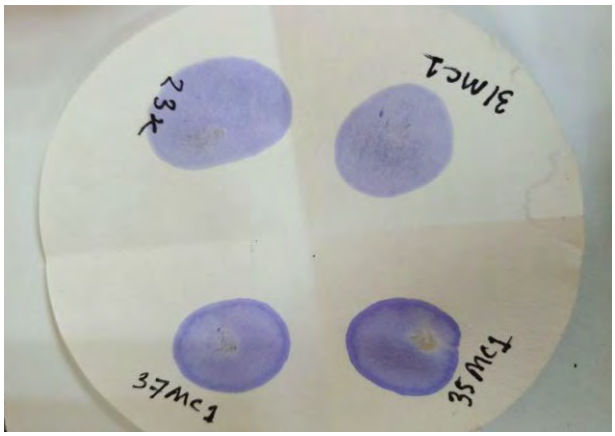


Figure 2.7: Oxidase test

2.8.6 Methyl Red (MR) Test

Methyl Red or MR test is used to identify the different types of facultative anaerobic enteric bacteria depending on their ability to stabilize acid end-product formation during glucose fermentation. Organisms that utilize a mixed-acid fermentation pathway that produces different organic acids such as lactic acid, acetic acid, formic acid etc. can be identified using this test. These acids lower the pH of the medium, creating an acidic environment in the test broth. This test along with Voges-Proskauer or VP test uses MRVP broth. After bacterial inoculation and incubation, an indicator Methyl Red is added to the broth to determine acid production. If the bacteria organism produces stable acid end products, the pH value becomes acidic thus turning the methyl red indicator red and suggesting a positive result. However, if a stable acid end product was not formed

by the organism, the broth loses acidity, raising its pH and thus turning the methyl red indicator yellow and suggesting a negative result. The observation and test results are stated in the table below:

Observation	Result	Example
Red color	Positive	*E. coli*
Yellow color	Negative	*Enterobacter aerogenes*

Table 2.9: Methyl Red test

Procedure

1. The MRVP broth is inoculated with a pure culture of the desired organism.
2. The broth is incubated at 37°C for 48 hours.
3. 5-6 drops of methyl red reagent were added to per 5 mL of broth.
4. Color change in the medium was observed.

2.8.7 Voges Proskauer (VP) Test

The Voges Proskauer or VP test is used to determine an organism's ability to metabolize pyruvate into acetoin, a neutral end product. The VP test was introduced by Voges and Proskauer in 1898 which was later modified by Barritt in 1936 by introducing Barritt's reagents containing α-naphthol and KOH. Bacteria that can ferment glucose through the butylene glycol pathway produce acetoin. In the presence of oxygen and KOH, the acetoin oxidizes to diacetyl. In the presence of α-naphthol, the diacetyl then reacts with the guanidine component of peptone and forms a pink to red colored complex, thus confirming acetoin production and showing a VP positive result. In contrast, if a pink or red color is not formed then the determined result is VP negative. The reaction is as follows:

3.3 PCR Results

Control			
Sl. No	Isolate	PCR result	Sl.
1	C24MS1	Staphylococcus aureus	1

Glucose – (Glycolysis) → Pyruvate – (butanediol pathway) → Acetoin
Acetoin – (Oxygen, KOH) → Diacetyl + H_2O
Diacetyl + guanidine component of peptone → (alpha-naphthol) → Pink/red color

The observations and results are shown in the table below:

Observation	Result	Example
Red color	Positive	*Klebsiella pneumoniae*
Yellow color	Negative	*E. coli*

Table 2.10: Voges Proskauer test

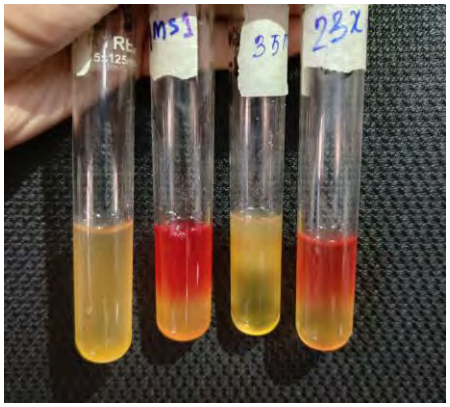


Figure 2.8: Voges Proskauer and Methyl Red test

2.8.8 Motility Test

Motility is the ability of an organism to move and glide by itself using special structures such as flagella and fibrils. Flagella act like propellers for movement, they are thread-like structures that extend from the organisms' membrane and cell wall. This characteristic can distinguish microorganisms that hold such structures such as Enterobacteriaceae family from other microorganisms. The motility test is done using a semi-solid agar medium so that bacterial movement can be observed by the naked eye. The soft consistency of the medium allows motile

Sample	
Isolate	PCR result
S4MS1	Staphylococcus aureus

organisms to migrate through it and create cloudiness in the medium. Bacteria is inoculated in the medium using a straight needle, when it is motile, it will show a diffuse zone of growth that extends from the line of inoculation. However, the non-motile bacteria will only grow along the stab line.

Procedure:

1. A semi solid media is prepared and poured into vials
2. A colony of a 24-hour fresh culture is touched using a straight needle to
3. The media is stabbed only one time, making sure to keep the needle in the same line it entered as it is removed from the medium.
4. It is incubated at 37°C and observed for a diffuse zone of growth flaring out from the line of inoculation.

2.8.9 Urease Test

The urease test determines the ability of an organism to break down urea using the urease enzyme. Urea is the product of decarboxylation of amino acids. Urea breaks down into ammonia, carbon dioxide and water through hydrolysis catalyzed by urease enzyme. The reaction is as follows:

$$\text{Urea} + 2H_2O - (\text{Urease}) \rightarrow \text{Carbon dioxide} + \text{Water} + \text{Ammonia}$$

The resulting ammonia increases the pH of the medium, thus causing the phenol red indicator in the medium to change color. Urease-positive organisms cause the media to turn pink from yellow as it rapidly break down urea and increases the pH of the media. On the other hand, urease-negative organisms do not produce ammonia, thus not changing the color of the medium.

Urease-positive microorganisms: *Proteus spp., Cryptococcus spp. etc.*

Urease negative microorganisms: *Escherichia, Shigella, Salmonella, etc.*

Figure 2.9: Urease positive, Motile microorganism

2.8.10 Indole Test

An indole test is performed to identify the ability of a microorganism to break down tryptophan using tryptophanase, into indole. Pyruvate and ammonia. During the test, tryptophan undergoes deamination and tryptophanase removes the amine group (-NH2) from tryptophan, thus forming indole, pyruvate, and ammonium. The reaction is as follows:

Tryptophan + H_2O – (Tryptophanase) → Indole + Pyruvate + Ammonium

Kovac's reagent is added to the medium which contains hydrochloric acid and p-dimethylaminobenzaldehyde dissolved in amyl alcohol that reacts with indole if present and causes a visible ring in red color to form at the top of the broth medium, representing a positive result. When a ring does not form at the top, it indicates a negative result.

Indole-positive microorganisms: *Bacillus alvei, Edwardsiella sp., Escherichia coli etc.*

Indole-negative microorganisms: *Klebsiella spp., Neisseria spp.*

2.9. Isolation of Bacterial DNA

Isolation of DNA is essential prior to PCR and Gel electrophoresis in order to retrieve pure DNA from the colonies. DNA extraction ensures the removal of proteins, cell wall and other cellular components that could interfere when performing PCR to accurately visualize the bands in gel electrophoresis. For DNA isolation, the boiling method was used.

Procedure:

1. 150ml of TE buffer is taken into a microcentrifuge tube.
2. A loopful of the desired colony is taken from the 24hrs fresh culture and mixed into the buffer.
3. The colony is mixed using a vortex machine.
4. The samples are heated using a heat block at 95°C for 20 minutes.
5. The samples are then centrifuged at 4°C and 10000 rpm for 10 minutes.
6. The debris fall to the bottom and the pure DNA remains suspended in the supernatant.
7. Supernatant is collected for further procedures.

2.10 Polymerase Chain Reaction (PCR) Test

Polymerase Chain Reaction or PCR was introduced by Mullis in the year 1983. PCR is a powerful tool for DNA purification as well as DNA cloning as it allows replication of specific DNA sequences in vitro and thus produces billions of copies from a trace amount of DNA using DNA polymerase.

The elements needed for PCR are:

1. **DNA template: A** DNA template is the desired DNA sequence to be replicated which can be genomic, complementary (cDNA), or mitochondrial DNA and messenger RNA (mRNA).

2. **Primers:** Primers are short DNA or RNA sequences that mark the starting points for DNA synthesis by binding to the DNA template. Complementary primers are used in the process as forward and reverse primers for forward and reverse strands.
3. **dNTPs or DNA Nucleotide Bases:** The dNTPs are building blocks of the new strand to be synthesized, such as adenine (A), thymine (T), cytosine (C) and guanine (G).
4. **Taq polymerase:** Taq polymerase is an enzyme that is heat-stable and adds nucleotide bases to new DNA strands. It is derived from a thermophile called Thermus aquaticus.
5. **Buffer:** Buffer maintains the right pH and ionic strength and thus keeps the reaction under optimal conditions.

There are 5 stages of the PCR cycle which are:

1. **Denaturing:** The double-stranded DNA gets denatured in this step and produces a single-stranded DNA, ready to be amplified. This reaction occurs at 94-98°C for initially 1-3 minutes and finally 30 seconds in each cycle.
2. **Annealing:** Primers anneal to their complementary sequences on the denatured single-stranded templates in this step. The temperature is brought down to around 50-65°C depending on the melting point of the primers used and runs for 20-40 seconds each cycle.
3. **Elongation:** During elongation, nucleotides are added to the primers to synthesize the new strands with the help of DNA polymerase. The temperate is kept at 72°C for 30-60 seconds each cycle.
4. **Extension:** This stage occurs at the end of the cycles which is around 5-10 minutes long at the same temperature as the previous step. This step ensures that all the remaining single-stranded DNA is fully extended to complete double-stranded products.

5. **Final Hold:** The PCR products can be held at 4°C to preserve the PCR products until they are used in further experiments.

For this study, 2µL of the desired DNA template, 6.5µL master mix, 2.5µL of nuclease-free water and 1µL each of forward and reverse primer were added to make a total of 13 µL reaction mixture. The conditions used for each gene primer are given in the table below:

Name of the Microorganism	Primer used	Primer Sequence	PCR Conditions	No. of Cycles	Product Size
Staphylococcus aureus	NucA	**F:**5'GCGATTGATGGTGATACGGT-3' **R:**5'AGCCAAGCCTTGACGAACTAAAGC-3'	95°C for 5 min 95°C for 1 min 60°C for 30 sec 72°C for 1 min. 72°C for 10 min	30	279bp
Staphylococcus spp.	TStaG	**F:**5′GGCCGTGTTGAACGTGGTCAAATCA -3′ **R**:5′TIACCATTTCAGTACCTTCTGGTAA-3′	94°C for 5 min 94°C for 30 sec 55°C for 30sec 72°C for 30sec 72°C for 5 min	30	370bp

Table 2.11: Primers used for PCR

2.11 Gel electrophoresis

Gel electrophoresis is a technique in molecular biology that is used to separate DNA, RNA, or proteins depending on their charge and size. A gel is used in this process which is made of a porous material such as agarose for DNA and RNA or polyacrylamide gel for proteins. The gel is placed into an electrophoresis chamber and flooded with buffer, upon which desired molecules or mostly the PCR products are placed in the wells made into the gel. An electric current is applied thus making the molecules migrate through the gel from one end to another, usually for DNA to move towards the positive end as DNA itself is negatively charged. The size of the pores in the gel determines how far the molecules will travel and through a pre-determined ladder comparison, the bands can be measured for their size. On the other hand, the different stains such as ethidium bromide used for visualization of the bands allow the spotting of the band through their color intensity when put under a UV light.

2.12 Antibiotic Resistance and Susceptibility Analysis

The antibiotic susceptibility test can give results to three different categories which are Susceptible, Resistant, and Intermediate.

2.12.1 List of Antibiotics and their Zone of Inhibition:

Antibiotic	Zone of inhibition (mm)		
	Susceptibility	Intermediate	Resistance
Methicillin (MET)	≥14	10-13	≤9
Cefepime (FEP)	≥18	15-17	≤14
Doxycycline Hydrochloride (DO)	≥14	11-13	≤10
Gentamicin (GEN)	≥15	13-14	≤12
Amoxicillin (AMX)	≥18	14-17	≤13
Erythromycin (E)	≥23	14-22	≤13
Vancomycin (VAN)	≥12	10-11	≤9
Penicillin (P)	≥28	20-27	≤19
Levofloxacin (LEV)	≥17	14-16	≤13
TGC	≥15	12-14	≤11
Cefixime (CFM)	≥19	16-18	≤15
Azithromycin (AZM)	≥18	14-17	≤13
Amikacin (AK)	≥17	15-16	≤14
Ampicillin (AMP)	≥17	14-16	≤13
Piperacillin (PIT)	≥18	-	≤17
Cefepime (CPM)	≥18	15-17	≤14
Gentamicin (CN)	≥15	13-14	≤12

Table 2.12: Zone of Inhibition of the antibiotics used

2.12.2 Preparation of Mueller Hinton Agar

Mueller and Hinton invented Mueller Hinton Agar or MHA in 1941 to isolate the harmful bacteria Neisseria. Currently, it is an essential media used for antibiotic sensitivity testing through the Kirby-Bauer disk diffusion method. The nutrient source of the media is beef extract and acid hydrolysate of casein. Another important ingredient is starch which takes up harmful byproducts and releases dextrose upon breakdown which works as the energy source. MHA agar is highly functional for this test as it has a low level of Para-aminobenzoic acid and thymidine thus not interfering with the functions of antibiotics that show sulfonamide activities.

2.12.3 Bacterial Suspension Preparation, Inoculation and Disc Diffusion

1. 5 mL of sterile saline is added to a sterile test tube.
2. Selected colonies from fresh subculture are taken using an inoculating loop and transferred to the saline tube.
3. The colony is diluted to a turbidity of 0.5 McFarland test standard.
4. A sterile swab is dipped into the inoculum within 15 minutes of dilution.
5. The entire surface of the prepared MHA plate is streaked three times with the swab, turning the plate 90 degrees each time.
6. The antibiotic disks are placed with a sterile tweezer, keeping them at least 24mm apart, thus allowing a zone to grow to determine the correct result.
7. Plates are incubated at 37°C for exactly 24 hours.
8. The results are observed and zones are measured for further analysis.

Chapter 3: Results

The results are organized into four sections, with separate tables for the samples(20) and controls(40). A total of 78 isolates were identified in the control group, while the sample group contained 54 isolates. Section 3.1 presents the growth of the isolates in various media, Section 3.2 includes biochemical results and potential species identification, Section 3.3 covers the PCR results, and Section 3.4 details the antibiotic susceptibility test results along with the resistance patterns of the isolates.

3.1 Results of Growth on Selective Media

Result of the samples:

Isolate name	Sample No	Growth Medium	Color of the colony	Color of Media	Color on Hichrome UTI Media
S1MC1	1	MAC	Pink	Pink	Green
S1MS1	1	MSA	Yellow	Yellow	Brown
S2X1	2	XLD	white	Brown	Brown
S2X2	2	XLD	yellow	Brown	Brown
S2MC1	2	MAC	pink	Pink	No growth
S2MS1	2	MSA	yellow	Yellow	Brown
S3X1	3	XLD	white	Brown	Brown
S3X2	3	XLD	yellow	Brown	Brown
S3MC1	3	MAC	pink	Pink	Blue
S4MS1	4	MSA	White	Red	Purple
S4MS2	4	MSA	Yellow	Yellow	Brown
S5MS1	5	MSA	Yellow	Yellow	Brown
S6MS1	6	MSA	White	Red	Purple
S6MS2	6	MSA	Yellow	Red	White
S6MC1	6	MAC	pink	Pink	Blue
S6X1	6	XLD	yellow	Brown	No growth
S7MS1	7	MSA	White	Red	Purple
S7MC1	7	MAC	pink	Pink	Green
S8MS1	8	MSA	Yellow	Yellow	Brown

Isolate name	Sample No	Growth Medium	Color of the colony	Color of Media	Color on Hichrome UTI Media
S8X1	8	XLD	yellow	Brown	Brown
S8X2	8	XLD	white	Brown	Brown
S8MC1	8	MAC	pink	Pink	Brown
S9MS1	9	MSA	Orange	Yellow	Brown
S9MS2	9	MSA	white	Red	White
S9MC1	9	MAC	pink	Pink	No growth
S10MC1	10	MAC	pink	Light Pink	Blue
S10MS1	10	MSA	Yellow	Yellow	Brown
S10X1	10	XLD	pink	Pink	Blue
S11X1	11	XLD	Pink	Pink	No growth
S11MS1	11	MSA	Yellow	Yellow	Brown
S11MC1	11	MAC	transparent	Orange	No growth
S12MS1	12	MSA	Yellow	Yellow	Light Purple
S13X1	13	XLD	Yellow	Brown	No growth
S14MS1	14	MSA	Yellow	Yellow	Cream
S14MS2	14	MSA	Yellow	Red	Cream
S15MS1	15	MSA	Yellow	Yellow	Cream
S15MS2	15	MSA	White	Red	White
S15MS3	15	MSA	Pink	Red	White
S15MC1	15	MAC	Pink	Pink	Blue
S16MS1	16	MSA	Yellow	Yellow	Yellow
S16MS2	16	MSA	White	Red	Purple
S16MS3	16	MSA	Pink	Red	White
S17MS1	17	MSA	Yellow	Yellow	Yellow
S17MS2	17	MSA	White	Red	White
S17MS3	17	MSA	Pink	Red	White
S18MS1	18	MSA	White	Red	White
S18MS2	18	MSA	Yellow	Red	White
S19MS1	19	MSA	Yellow	Yellow	Yellow
S19MS2	19	MSA	White	Red	White
S19MS3	19	MSA	Pink	Red	White
S20MS1	20	MSA	Yellow	Yellow	Brown
S20MS2	20	MSA	White	Red	Purple
S20MS3	20	MSA	Pink	Red	White
S20MC1	20	MAC	Pink	Pink	No growth

Table 3.1: Results of Growth on Selective Media (Sample)

Result of the Controls:

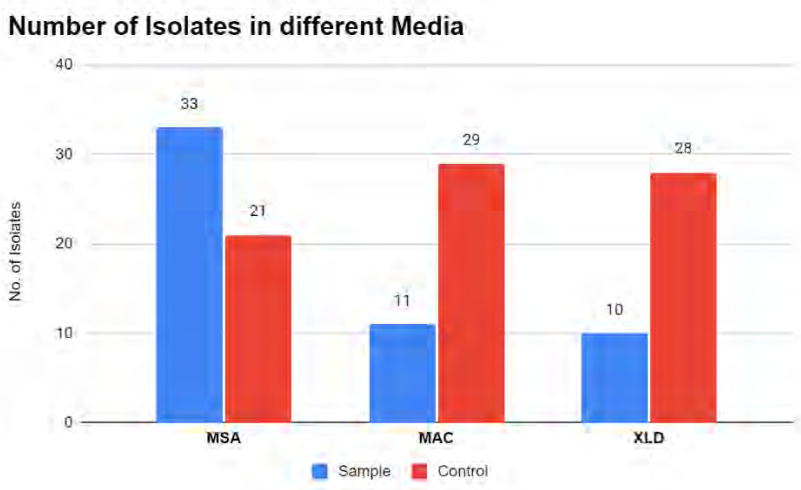
Isolate name	Sample No	Growth Medium	Color of the colony	Color of Media	Color on Hichrome UTI Media
C21MS1	21	MSA	White	Yellow	White
C21MC1	21	MAC	Pink	Orange	Blue
C22MC1	22	MAC	Pink	Pink	Blue
C22X1	22	XLD	Orange	Brown	No growth
C23MC1	23	MAC	Pink	Pink	No growth
C23X1	23	XLD	White	Pink	Brown
C24MS1	24	MSA	Yellow	Yellow	Brown
C24MC1	24	MAC	transparent	Orange	No growth
C24X1	24	XLD	Yellow	Pink	Cream
C25MC1	25	MAC	Pink	Orange	Green
C25X1	25	XLD	Orange	Pink	No growth
C26MS1	26	MSA	White	Red	Brown
C26MC1	26	MAC	transparent	Orange	No growth
C27MS1	27	MSA	White	Red	Brown
C27X1	27	XLD	White	Pink	Brown
C28MC1	28	MAC	Pink	Pink	Blue
C28X1	28	XLD	White	Pink	Brown
C29MC1	29	MAC	transparent	Orange	No growth
C29X1	29	XLD	White	Pink	Brown
C30MS1	30	MSA	White	Red	Brown
C30X1	30	XLD	Yellow	Yellow	Brown
C31MS1	31	MSA	White	Red	Brown
C31MC1	31	MAC	Pink	Pink	Green
C31X1	31	XLD	Yellow	Yellow	Brown
C32MC1	32	MAC	Pink	Orange	Blue
C32X1	32	XLD	small white	Pink	Brown
C33MC1	33	MAC	Pink	Pink	Green
C33X1	33	XLD	White	Pink	Brown
C34MC1	34	MAC	Pink	Orange	Green
C35MS1	35	MSA	Yellow	Yellow	Brown
C35MC1	35	MAC	Pink	Pink	Green
C35X1	35	XLD	White	Pink	Brown
C36MS1	36	MSA	Yellow	Yellow	Brown

Isolate name	Sample No	Growth Medium	Color of the colony	Color of Media	Color on Hichrome UTI Media
C36MC1	36	MAC	Pink	Orange	Blue
C36X1	36	XLD	White	Pink	Cream
C37MC1	37	MAC	transparent	Orange	No growth
C37X1	37	XLD	White	Pink	Brown
C38MS1	38	MSA	Yellow	Yellow	Brown
C38X1	38	XLD	White	Pink	Brown
C39MC1	39	MAC	Pink	Orange	Blue
C39X1	39	XLD	White	Pink	Brown
C40MS1	40	MSA	Yellow	Yellow	Brown
C40MC1	40	MAC	Pink	Pink	Green
C40X1	40	XLD	White	Pink	Brown
C41X1	41	XLD	White	Pink	Brown
C42MS1	42	MSA	Yellow	Yellow	Orange Brown
C42MC1	42	MAC	pink	Pink	Green Blue
C42X1	42	XLD	white	Orange	Brown
S43MS1	43	MSA	Yellow	Yellow	Orange Brown
S44MS1	44	MSA	white	Red	Brown
S44MS2	44	MSA	yellow	Yellow	Orange Brown
C45MS1	45	MSA	White	Red	Brown
C45X1	45	XLD	white	Orange	Brown
C46MC1	46	MAC	pink	Orange	Green Blue
C46X1	46	XLD	yellow	Orange	Orange Brown
C47MC1	47	MAC	pink	Pink	Green Blue
C48MS1	48	MSA	yellow	Yellow	Orange Brown
C49X1	49	XLD	yellow	Orange	Orange Brown
C50X1	50	XLD	yellow	Orange	Orange Brown
C51MS1	51	MSA	yellow	Yellow	Cream
C51MC1	51	MAC	Pink	Pink	Green Blue
C52MC1	52	MAC	pink	Pink	Green Blue
C53MS1	53	MSA	white	Yellow	Brown
C53MC1	53	MAC	pink	Pink	Green Blue
C53X1	53	XLD	white	Pink	Brown
C54MS1	54	MSA	white	Yellow	Brown
C55MS1	55	MSA	White	Yellow	Brown
C55MC1	55	MAC	Pink	Orange	Green Blue
C56MC1	56	MAC	pink	Pink	Green Blue
C56X1	56	XLD	orange	Orange	No growth

Isolate name	Sample No	Growth Medium	Color of the colony	Color of Media	Color on Hichrome UTI Media
C57MC1	57	MAC	pink	Pink	Green Blue
C57X1	57	XLD	white	Pink	Brown
C58MC1	58	MAC	transparent	Orange	Green Blue
C58X1	58	XLD	white	Pink	Brown
C59MS1	59	MSA	transparent	Pink	No growth
C59MC1	59	MAC	transparent	Orange	No growth
C59X1	59	XLD	pink	Pink	Green Blue
C60MC1	60	MAC	pink	Pink	Green Blue

Table 3.2: Results of Growth on Selective Media (Control)

The data related to the number of isolates found in different culture media for samples and controls are given in the graph below:



Graph 1: Number of isolates in differential media

MSA denotes for Mannitol Salt Agar Media

MAC denotes for Macconkey Agar Media

XLD denotes for Xylose Lysine Deoxycholate Agar Media

3.2 Identification of Isolates based on biochemical test results

Result of Samples:

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/ Black precipitate						Motility	Indole	Urease	Color	Shape	
S1MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
S1MS1	Yellow	Yellow	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S2X1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
S2X2	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Klebsiella spp.*
S2MC1	Red	Red	Black Precipitate	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
S2MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S3X1	Yellow	Yellow	None	Negative	Negative	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
S3X2	Yellow	Red	None	Negative	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Enterobacter spp.*
S3MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Red	*Klebsiella Pneumoniae*
S4MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S4MS2	pink	Red	None	Negative	Positive	Positive	Positive	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S5MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S6MS1	Yellow	Red	None	Negative	Positive	Positive	Positive	Positive	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S6MS2	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S6MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Red	*Klebsiella Pneumoniae*
S6X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Red	*Klebsiella spp.*
S7MS1	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S7MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
S8MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S20MC1	Red	Red	Black precipitate	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/Black precipitate						Motility	Indole	Urease	Color	Shape	
S8X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Enterobacter spp.*
S8X2	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
S8MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella pneumoniae*
S9MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S9MS2	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S9MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella pneumoniae*
S10MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella pneumoniae*
S10MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S10X1	Yellow	Yellow	Gas	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
S11X1	Yellow	Yellow	Gas	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
S11MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S11MC1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Salmonella typhimurium*
S12MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S13X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Enterobacter spp.*
S14MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S14MS2	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S15MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S15MS2	Yellow	Red	None	Negative	Positive	Positive	Positive	Positive	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/ Black precipitate						Motility	Indole	Urease	Color	Shape	
S15MS3	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*
S15MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella pneumoniae*
S16MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S16MS2	Yellow	Red	None	Negative	Positive	Positive	Positive	Positive	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S16MS3	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*
S17MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S17MS2	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S17MS3	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*
S18MS1	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S18MS2	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S19MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S19MS2	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S19MS3	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*
S20MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
S20MS2	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Yellow	Purple	Cocci	*Coagulase-negative staphylococci (CoNS)*
S20MS3	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*

Table 3.3: Results of Biochemical tests and identification of probable species (Sample)

Result of Controls:

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/ Black precipitate						Motility	Indole	Urease	Color	Shape	
C21MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C21MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Spp.*
C22MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C22X1	Yellow	Red	None	Positive	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C23MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C23X1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Positive	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C24MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C24MC1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Salmonella typhimurium*
C24X1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Enterobacter spp.*
C25MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Spp.*
C25X1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C26MS1	Yellow	Red	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C26MC1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Salmonella typhimurium*
C27MS1	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C27X1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C28MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C28X1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/Black precipitate						Motility	Indole	Urease	Color	Shape	
C29MC1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Salmonella typhimurium*
C29X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C30MS1	Yellow	Red	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C30X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Enterobacter spp.*
C31MS1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Purple	Cocci	*Staphylococci Spp.*
C31MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C31X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Enterobacter spp.*
C32MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Enterobacter spp.*
C32X1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C33MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C33X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C34MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Spp.*
C35MS1	Yellow	Red	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
C35MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C35X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C36MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
C36MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Enterobacter spp.*

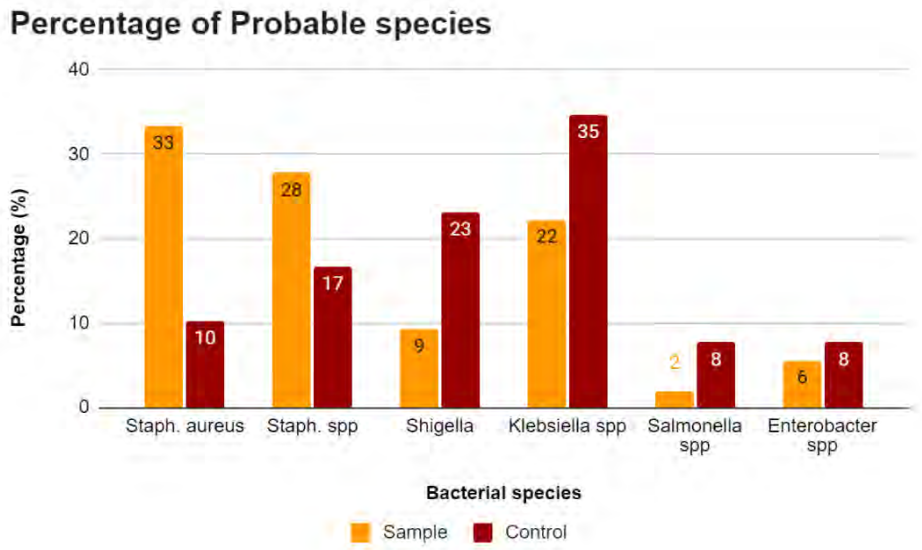
Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/Black precipitate						Motility	Indole	Urease	Color	Shape	
C36X1	Yellow	Red	None	Negative	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C37MC1	Red	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Salmonella typhimurium*
C37X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C38MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Pink	Rod	*Staphylococcus aureus*
C38X1	Yellow	Red	None	Negative	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C39MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Spp./*
C39X1	Yellow	Red	None	Negative	Positive	Negative	Positive	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C40MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus aureus*
C40MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C40X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Non-motile	Negative	Pink	Pink	Rod	*Shigella Spp.*
C41X1	Yellow	Red	None	Positive	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C42MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C42MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C42X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Enterobacter spp.*
S43MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Pink	*Staphylococcus aureus*
S44MS1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Pink	*Staphylococci Spp.*
S44MS2	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Pink	*Staphylococcus aureus*
C45MS1	Yellow	Yellow	None	Negative	Positive	Positive	Negative	Negative	Motile	Negative	Pink	Purple	Pink	*Staphylococci Spp.*
C45X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C46MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Klebsiella Spp./ enterobacter aerogenes*

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/Black precipitate						Motility	Indole	Urease	Color	Shape	
C46X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C47MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C48MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C49X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C50X1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C51MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C51MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C52MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C53MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococci Spp.*
C53MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C53X1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C54MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus Aureus*
C55MS1	Yellow	Yellow	None	Negative	Positive	Negative	Positive	Positive	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus Aureus*
C55MC1	Red	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Klebsiella Spp.*
C56MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Yellow	Pink	Rod	*Klebsiella Pneumoniae*
C56X1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella spp.*
C57MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*
C57X1	Yellow	Yellow	None	Positive	Positive	Negative	Positive	Negative	Motile	Negative	Pink	Pink	Rod	*Shigella Spp.*
C58MC1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Salmonella typhimurium*

Isolate name	BIOCHEMICAL TEST RESULTS													Probable species
	TSI			Citrate	Catalase	Oxidase	MR	VP	MIU			Gram Stain		
	Color of Slant	Color of Butt	Gas formation/Black precipitate						Motility	Indole	Urease	Color	Shape	
C58X1	Yellow	Yellow	None	Positive	Positive	Negative	Positive	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C59MS1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Purple	Cocci	*Staphylococcus epidermidis*
C59MC1	Yellow	Yellow	None	Positive	Positive	Negative	Negative	Negative	Motile	Negative	Pink	Pink	Rod	*Salmonella typhimurium*
C59X1	Yellow	Yellow	None	Negative	Positive	Negative	Negative	Negative	Motile	Negative	Yellow	Pink	Rod	*Shigella Spp.*
C60MC1	Yellow	Red	None	Negative	Positive	Negative	Negative	Negative	Non-motile	Negative	Pink	Pink	Rod	*Klebsiella Pneumoniae*

Table 3.4: Results of Biochemical tests and identification of probable species (Control)

Percentage of the identified isolates from samples and controls:



Graph 2: Percentage of probable species

ed color is not formed then the determined result is VP

microorganisms that hold such s

microorganisms. The motility test

movement can be observed by the n

3.3 PCR Results

Control			Sample		
Sl. No	**Isolate**	**PCR result**	**Sl. No**	**Isolate**	**PCR result**
1	C24MS1	Staphylococcus aureus	1	S4MS1	Staphylococcus aureus
2	C35MS1	Staphylococcus aureus	2	S5MS1	Staphylococcus aureus
3	C38MS1	Staphylococcus aureus	3	S8MS1	Staphylococcus aureus
4	C40MS1	Staphylococcus aureus	4	S9MS1	Staphylococcus aureus
5	S43MS1	Staphylococcus aureus	5	S10MS1	Staphylococcus aureus
6	C51MS1	Staphylococcus aureus	6	S11MS1	Staphylococcus aureus
7	C26MS1	*Staphylococcus spp.*	7	S12MS1	Staphylococcus aureus
8	C27MS1	*Staphylococcus spp.*	8	S16MS1	Staphylococcus aureus
9	C30MS1	*Staphylococcus spp.*	9	S20MS1	Staphylococcus aureus
10	C31MS1	*Staphylococcus spp.*	10	S2MS1	*Staphylococcus spp.*
11	S44MS1	*Staphylococcus spp.*	11	S4MS2	*Staphylococcus spp.*
12	S44MS2	*Staphylococcus spp.*	12	S6MS1	*Staphylococcus spp.*
13	C45MS1	*Staphylococcus spp.*	13	S6MS2	*Staphylococcus spp.*
14	C54MS1	*Staphylococcus spp.*	14	S7MS1	*Staphylococcus spp.*
15	C55MS1	*Staphylococcus spp.*	15	S14MS1	*Staphylococcus spp.*
			16	S14MS2	*Staphylococcus spp.*
			17	S15MS1	*Staphylococcus spp.*
			18	S15MS2	*Staphylococcus spp.*
			19	S15MS3	*Staphylococcus spp.*
			20	S16MS2	*Staphylococcus spp.*
			21	S16MS3	*Staphylococcus spp.*
			22	S17MS2	*Staphylococcus spp.*
			23	S18MS1	*Staphylococcus spp.*
			24	S18MS2	*Staphylococcus spp.*
			25	S19MS1	*Staphylococcus spp.*
			26	S19MS2	*Staphylococcus spp.*
			27	S19MS3	*Staphylococcus spp.*
			28	S20MS2	*Staphylococcus spp.*

Table 3.5: PCR Result

PCR was performed to confirm the isolates of *staphylococcus aureus* and other *staphylococcus* species using the specific primers and following the protocols mentioned in sections 2.9 and 2.10. The PCR products were then put into gel electrophoresis where 1.5% agarose gel was used. The picture were taken in the presence of UV light to observe the bands.

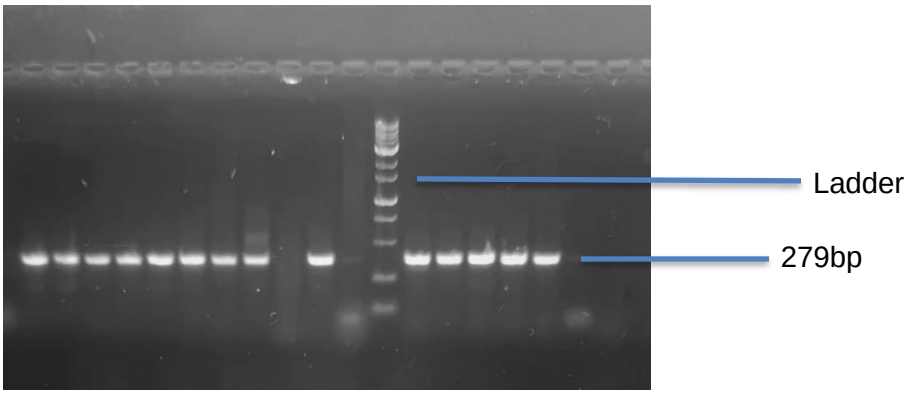


Figure 3.1: Gel electrophoresis for *Staphylococcus aureus*

The figure shows the Gel electrophoresis of the PCR products for *Staphylococcus aureus*. Here, a 100bp ladder was used. Thus, the bands confirm the species to be *Staphylococcus aureus* with a band length of 279bp. The PCR results confirmed that 6 isolates from the control group and 9 isolates from the sample group were *Staphylococcus aureus*.

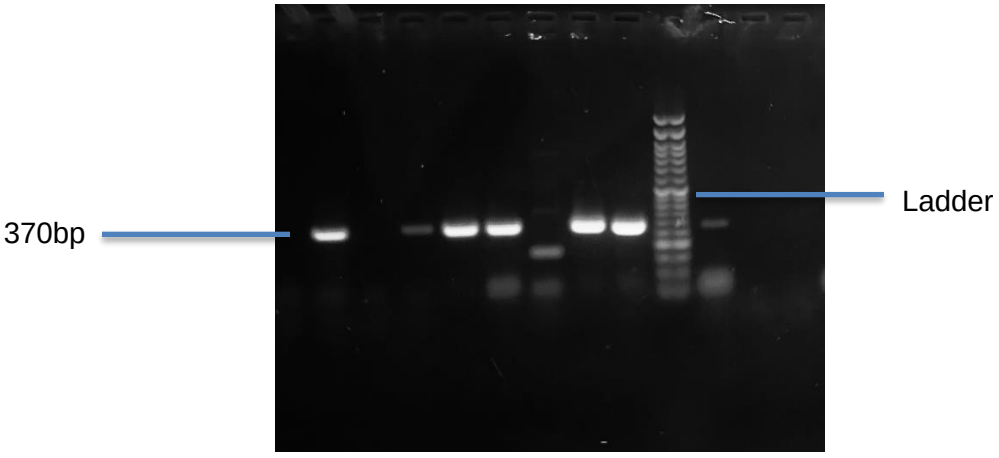
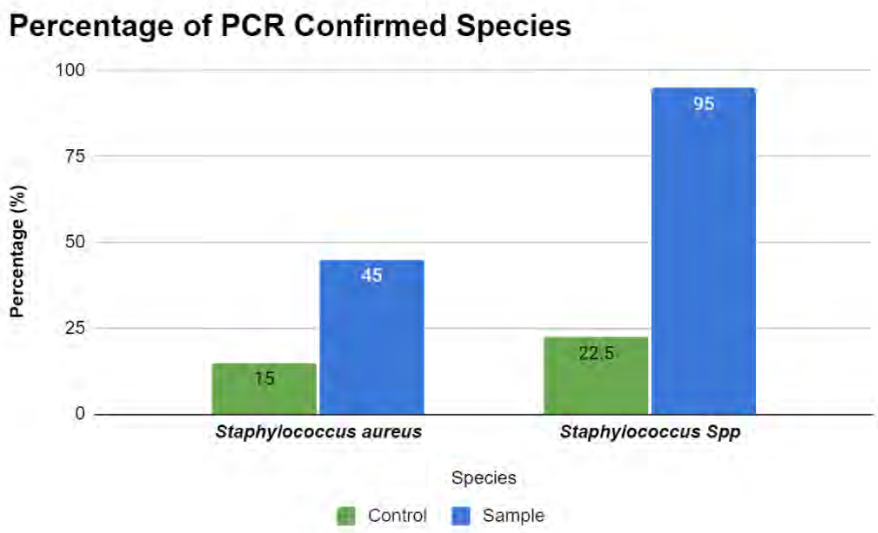


Figure 3.2: Gel electrophoresis for *Staphylococcus* species

The figure shows the Gel electrophoresis of the PCR products for *Staphylococcus* species. Here, a 50bp ladder was used. Thus, the bands confirm the species to be *Staphylococcus* with a band length of 370bp. The PCR results confirmed that 9 isolates from the control group and 19 isolates from the sample group were *Staphylococcus spp.*.

The PCR results are shown in the graph below:



Graph 3: PCR results

The PCR test confirmed the presence of *Staphylococcus aureus* in 15% of the controls and in 22.5% of the samples. Moreover, Staphylococcus species were confirmed to be present in 45% of the controls and in 95% of the Samples.

3.4 Antibiotic Susceptibility Test results

R = Resistant; I = Intermediate; S = Sensitive

Isolate No	Antibiotic Susceptibility of Gram-Positive Isolates (Sample)									
	Methicillin (MET)	Cefepime (FEP)	Doxycycline Hydrochloride (DO)	Gentamicin (GEN)	Amoxicillin (AMX)	Erythromycin (E)	Vancomycin (VAN)	Penicillin (P)	Levofloxacin (LEV)	TGC
10MS1	R	R	R	S	R	R	R	R	R	R
12MS1	R	R	R	R	R	R	R	R	R	R
14MS1	R	S	S	S	R	S	S	R	R	S
14MS2	S	S	S	S	R	R	S	R	S	S
15MS1	R	S	S	S	R	R	R	R	S	I
15ms2	R	S	S	S	R	R	R	R	S	S
15MS3	S	S	S	S	R	R	S	R	S	S
16ms1	R	S	S	S	R	I	S	R	S	S
16Ms2	R	R	S	S	R	S	S	R	S	S
16MS3	R	S	R	S	R	R	R	R	R	I
17MS1	R	R	R	S	R	R	R	R	R	R
17MS2	R	S	R	S	R	R	R	R	R	I
18MS1	R	S	R	S	R	R	R	R	R	I
18MS2	R	S	R	S	R	R	R	R	R	I
19MS1	S	S	S	S	R	S	S	I	S	S
19MS2	R	S	S	S	R	S	S	R	I	S
19MS3	R	S	S	S	R	R	S	R	R	S
1M1	R	I	S	S	R	R	S	R	S	S
1MS1	R	R	S	S	R	R	S	R	R	S
20MS1	R	R	R	R	R	R	R	R	R	R
20MS2	R	R	S	S	R	R	S	R	S	S
20MS3	R	S	S	S	R	R	S	R	S	S
2ms1	R	I	S	S	R	R	R	R	S	S
4ms1	R	R	S	R	R	R	R	R	R	I

Isolate No	Antibiotic Susceptibility of Gram-Positive Isolates (Sample)									
	Methicillin (MET)	Cefepime (FEP)	Doxycycline Hydrochloride (DO)	Gentamicin (GEN)	Amoxicillin (AMX)	Erythromycin (E)	Vancomycin (VAN)	Penicillin (P)	Levofloxacin (LEV)	TGC
4MS2	R	S	R	S	R	R	R	R	R	R
5MS1	R	R	R	S	R	R	R	R	R	I
6MS1	R	S	R	S	R	R	R	R	R	I
6MS2	R	R	R	S	R	R	R	R	R	I
7MS1	R	S	R	S	R	R	R	R	R	I
8MS1	R	S	R	S	R	R	R	R	R	I
9MS1	R	S	R	S	R	R	R	R	R	I
9MS2	R	S	S	S	R	R	R	R	S	S

Table 3.6: Antibiotic Susceptibility of Gram-Positive Isolates (Samples)

Isolate No	Antibiotic Susceptibility of Gram-Positive Isolates (Controls)									
	Methicillin (MET)	Cefepime (FEP)	Doxycycline Hydrochloride (DO)	Gentamicin (GEN)	Amoxicillin (AMX)	Erythromycin (E)	Vancomycin (VAN)	Penicillin (P)	Levofloxacin (LEV)	TGC
21MS1	S	S	S	S	R	R	R	R	S	S
24MS1	R	R	S	S	R	S	S	R	R	S
25ms1	R	R	S	S	R	R	S	R	S	S
26MS1	R	S	S	S	R	R	R	R	S	S
27MS1	S	S	S	S	R	R	R	R	S	S
30MS1	R	I	R	S	R	R	R	R	R	I
31MS1	R	S	R	S	R	R	R	R	R	I
35MS1	R	R	R	S	R	R	R	R	R	S
36MS1	R	R	R	S	R	R	R	R	R	I
38MS1	R	S	S	S	R	R	R	R	S	S
40MS1	R	S	S	S	R	R	S	R	S	S
42MS1	R	S	R	S	R	R	R	R	S	I
43MS1	R	S	S	S	R	R	R	R	S	S
44ms1	R	S	R	S	R	R	R	R	R	I
44MS2	R	S	R	S	R	R	R	R	R	R
45MS1	R	S	S	S	R	R	S	R	S	S
51ms1	R	R	R	S	R	R	R	R	R	R
53ms1	R	I	I	S	R	R	R	S	R	S
54MS1	R	I	R	R	R	R	R	R	R	I
55MS1	R	R	R	S	R	R	R	R	R	I
59MS1	R	I	R	S	R	R	R	R	R	R

Table 3.7: Antibiotic Susceptibility of Gram-Positive Isolates (Control)

Isolate No	Antibiotic Susceptibility of Gram-Negative Isolates (Sample)									
	Cefepime (CPM)	Levofloxacin (LEV)	Doxycycline Hydrochloride (DO)	Cefixime (CFM)	Azithromycin (AZM)	Amikacin (AK)	Ampicillin (AMP)	Piperacillin (PIT)	Gentamicin (CN)	Amoxicillin (AMX)
10MC1	R	S	S	R	S	S	S	S	S	R
10X1	I	S	R	R	R	S	R	S	S	R
11MC1	R	S	S	R	R	S	R	S	S	R
11X1	I	S	S	R	S	S	R	S	S	R
13MC1	I	S	R	R	R	S	R	S	S	R
15MC1	I	S	S	R	S	S	R	S	S	R
20MC1	R	R	R	R	R	S	R	S	S	R
2MC1	I	R	R	I	R	S	R	S	R	R
3MC1	R	R	R	R	R	R	R	S	I	R
3X1	R	R	R	R	R	S	R	S	R	R
3X2	R	R	R	R	R	S	R	S	I	R
6MC1	S	S	S	R	S	S	S	S	S	R
6X1	R	S	S	R	I	S	R	S	S	R
7MC1	S	S	S	S	S	S	S	S	S	R
8MC1	R	S	S	R	I	S	R	S	S	R
8X1	S	S	S	S	S	S	S	S	S	R
8X2	R	R	S	R	R	S	R	S	R	R
9MC1	R	S	S	R	R	S	R	S	S	R
S1MC1	R	R	S	R	R	S	R	S	R	R
S2X1	R	R	S	R	R	S	R	S	R	R
S2X2	R	S	S	R	R	S	S	S	S	R

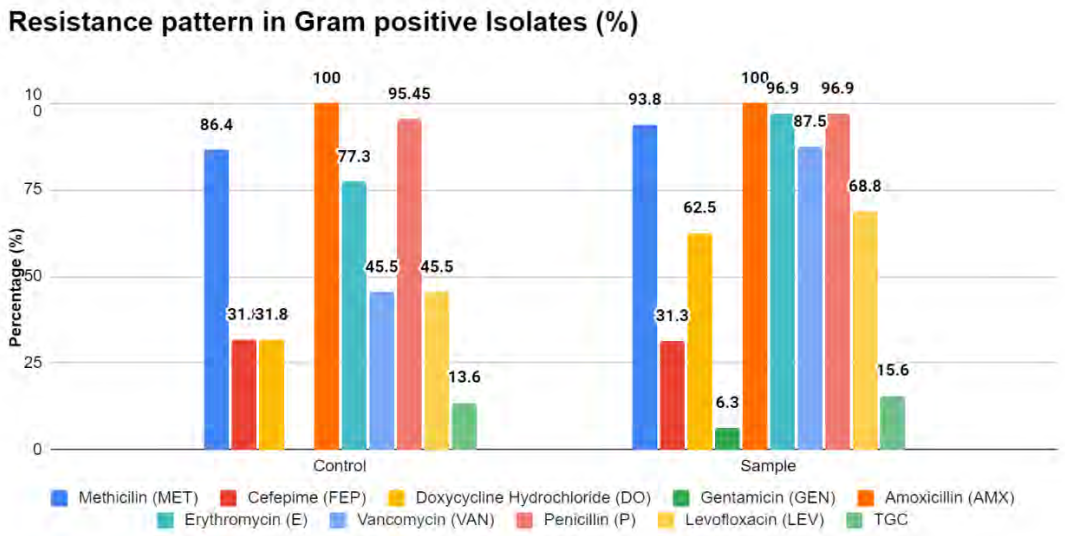
Table 3.8: Antibiotic Susceptibility of Gram-Negative Isolates (Sample)

Isolate No	Antibiotic Susceptibility of Gram-Negative Isolates (Control)									
	Cefepime (CPM)	Levofloxacin (LEV)	Doxycycline Hydrochloride (DO)	Cefixime (CFM)	Azithromycin (AZM)	Amikacin (AK)	Ampicillin (AMP)	Piperacillin (PIT)	Gentamicin (CN)	Amoxicillin (AMX)
21MC1	R	S	S	R	R	S	S	S	S	R
22MC1	S	S	I	S	R	S	R	S	S	R
22X1	R	S	S	R	S	S	R	S	S	R
23MC1	R	S	S	R	S	I	R	S	S	R
23X1	R	S	S	R	S	S	S	S	S	R
24MC1	I	S	R	R	R	S	R	S	S	R
24X1	R	S	S	R	R	S	S	S	S	R
25MC1	R	S	S	R	R	S	S	S	S	R
25X1	R	S	S	R	R	S	S	S	S	R
26MC1	R	S	S	R	R	S	R	R	S	R
27X1	I	S	S	R	S	S	S	S	S	R
28MC1	S	S	S	I	S	S	R	S	S	R
28X1	R	R	R	R	R	R	R	R	R	R
29MC1	S	S	I	S	R	S	R	S	S	R
29X1	I	S	S	R	R	S	R	S	S	R
30X1	R	R	R	R	R	S	R	S	S	R
31MC1	S	S	S	I	S	S	R	S	S	R
31X1	R	S	S	R	R	S	S	S	S	R
32MC1	R	R	R	R	R	S	R	S	R	R
32X1	I	S	R	R	R	S	R	S	S	R
33MC1	I	R	R	S	R	S	R	S	R	R
33X1	S	R	R	I	R	S	R	S	R	R
34MC1	S	S	S	I	S	S	R	S	S	R
35MC1	I	R	R	I	R	R	R	R	R	R
35X1	I	S	S	R	R	S	R	S	S	R
36MC1	R	R	R	R	R	S	R	R	R	R
36X1	R	S	S	R	R	S	S	S	S	R
37MC1	I	S	S	R	S	S	S	S	S	R
37X1	R	R	R	R	R	S	R	S	S	R
38X1	S	S	S	S	S	S	S	S	S	R
39MC1	S	S	R	S	R	S	R	S	S	R

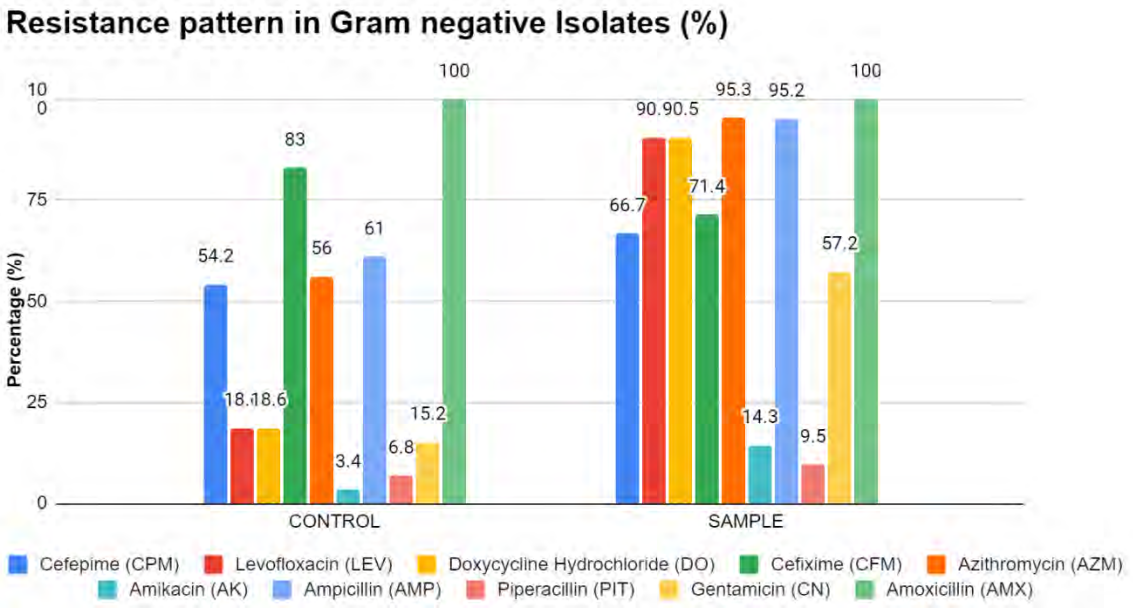
Isolate No	Antibiotic Susceptibility of Gram-Negative Isolates (Control)									
	Cefepime (CPM)	Levofloxacin (LEV)	Doxycycline Hydrochloride (DO)	Cefixime (CFM)	Azithromycin (AZM)	Amikacin (AK)	Ampicillin (AMP)	Piperacillin (PIT)	Gentamicin (CN)	Amoxicillin (AMX)
39X1	S	S	S	R	S	S	R	S	S	R
40MC1	S	R	R	S	R	S	R	S	I	R
40X1	S	S	S	S	S	S	S	S	S	R
41X1	R	S	S	R	R	S	R	S	S	R
42MC1	R	R	R	R	R	S	R	S	R	R
42X1	R	R	R	R	R	S	R	S	R	R
45X1	R	R	R	R	R	S	S	R	R	R
46MC1	R	R	S	R	R	S	R	S	R	R
46X1	I	R	S	R	R	S	R	S	R	R
47MC1	R	S	S	R	I	S	R	S	S	R
49X1	I	S	S	R	S	S	I	S	S	R
50X1	R	R	R	R	R	S	R	S	R	R
51MC1	R	S	S	R	R	S	S	S	S	R
52MC1	R	R	R	R	R	S	R	S	R	R
53MC1	S	S	S	R	S	S	S	S	S	R
53X1	R	S	S	R	S	S	S	S	S	R
55MC1	I	S	R	R	R	S	R	S	S	R
55MC1	I	S	S	R	S	S	R	S	I	R
56MC1	R	S	S	R	S	S	R	S	I	R
56MC1	R	S	S	R	I	S	R	S	S	R
56X1	R	S	S	R	R	S	S	S	S	R
57MC1	I	S	S	R	S	S	S	S	S	R
57X1	R	R	I	R	R	S	R	S	I	R
58MC1	R	R	R	R	R	R	R	R	R	R
58X1	S	R	R	S	R	I	S	S	I	R
59MC1	R	R	R	R	R	S	R	S	R	R
59X1	R	R	R	R	R	R	R	S	I	R
60MC1	R	R	R	R	R	S	R	S	R	R

Table 3.6: Antibiotic Susceptibility of Gram-Negative Isolates (Control)

The antibiotic resistance pattern is shown in two different graphs, one for gram-positive isolates and another for gram-negative isolates.



Graph 4: Antibiotic resistance pattern of Gram-Positive isolate



Graph 5: Antibiotic resistance pattern (Gram Negative)

Chapter 4: Discussion

The study showed a noteworthy pattern concerning the isolates retrieved from the control or neurotypical group and the sample or the group with neurodevelopmental disorders. Although there were twice the number of controls that of samples, the difference was not proportional in the case of the number of isolates found. A total of 78 isolates were found in the control group, whereas the number was 54 in the sample group, thus indicating a higher density of bacterial isolates within the sample group. Of these isolates, in the control group, gram-positive bacteria were 27% and gram-negative bacteria were 73%. Whereas, in the sample group, the isolates depicted 61% of gram-positive isolates and 39% gram-negative isolates. Of these isolates, the probable species identified in the sample were, 33% *Staphylococcus aureus*, 28% *Staphylococcus species*, 9% *Shigella species*, 22% *Klebsiella species*, 2% *Salmonella* species and 6% *Enterobacter* species. For the controls, the probable species identified were 10% *Staphylococcus aureus*, 17% *Staphylococcus species*, 23% *Shigella species*, 35% *Klebsiella* species, 8% *Salmonella* species and 8% *Enterobacter* species. The PCR analysis further confirmed the findings to be that the Staphylococcus species including Staphylococcus aureus were significantly more abundant in the sample group. Shockingly enough, the number of staphylococcus species found was twice the amount found in the control groups, leading to the concerning fact that the presence of *Staphylococcus aureus* was in 15% of the controls and in 22.5% of the samples. Moreover, other *Staphylococcus species* were confirmed to be present in 45% of the controls and in 95% of the Samples. This represents that children in the sample group who have neurodevelopmental disorders might be more vulnerable to bacterial colonization.

In addition to that, the antibiotic susceptibility pattern also highlights a concerning vulnerability among children with neurodevelopmental disorders in comparison to the control group, emphasizing their heightened resistance to several key antibiotics. For Gram-positive isolates, there was a somewhat similar resistance pattern, however, a stark difference was observed particularly for Vancomycin, Doxycycline, and Levofloxacin. The resistance pattern for Vancomycin was significantly higher in the sample group, with 87.5% of isolates showing resistance, compared to 45.5% in the control group. This elevated resistance poses serious challenges for treating infections in these children, as Vancomycin is often a last resort for multidrug-resistant Gram-positive infections (Garcia et al., 2019). Likewise, resistance to Doxycycline was nearly double in the sample group at 62.5% compared to the control group being 31.8%, and resistance to Levofloxacin was also significantly higher in the sample group with a percentage of 68.8% compared to controls at 45.5%.

The antibiotic resistance pattern observed in Gram-negative isolates was even more alarming. The sample group demonstrated a much higher resistance to most of the antibiotics. For instance, resistance to Levofloxacin and Doxycycline reached 90.5% in the sample group, while the control group showed much lower resistance rates of 18.6% for both antibiotics. This pattern was consistent for most other antibiotics as well, for example, resistance to Azithromycin, Ampicillin, and Gentamicin reached to 95.3%, 95.2%, and 57.2% in the sample group. On the contrary, the control group showed resistance rates of 56%, 61%, and 15.2% for the same antibiotics. These differences highlight the vulnerability of children with neurodevelopmental disorders to antibiotic treatment, as they are resistant to most antibiotics commonly used to treat bacterial infections. (Smith et al., 2023).

The causes of such differences in the results can be due to several factors. Firstly, a major factor that contributes to this case is the immune dysregulation that comes with neurodevelopmental disorders. Research suggests that children with conditions like autism spectrum disorder and cerebral palsy have compromised immune responses, thus making them more susceptible to infections and microbial colonization. (Brown et al., 2021) A study also shows that these abnormalities in the immune system can inhibit the ability of the person's body to clear bacterial pathogens which leads to higher rates of colonization by organisms such as Staphylococcus aureus and further support the growth and persistence of resistant bacteria. (Williams & Johnson, 2020) On the other hand, neurotypical individuals have a stronger immune system that is better equipped to regulate bacterial populations and prevent over-colonization. Additionally, their compromised immune systems may expose them to prolonged use of antibiotics, which can further contribute to the development of resistance. Repeated courses of antibiotics can disrupt the natural microbiome and encourage the survival of resistant bacteria, further exacerbating the issue. (Brown et al., 2021)

Moreover, another reason for having such distinct results could be due to the behavioral challenges that come with neurodevelopmental disorders. Such behaviors can be difficulty maintaining hygiene, repetitive behaviors, and selective eating habits, which may increase exposure to and retention of microbes. (Jones & Miller, 2022) The behavioral and environmental challenges these children face could also be contributing to this increased antibiotic resistance. These behavioral issues may increase their exposure to resistant strains and hinder the effective management of bacterial infections (Williams & Johnson, 2020). On the contrary, neurotypical children do not

show such behavior and practice maintaining regular hygiene routines, therefore minimizing the possible bacterial colonization and resistance pattern.

Furthermore, another contributing factor could be the increased use of medical devices and healthcare services that children with neurodevelopmental disorders need to get through. Children with neurodevelopmental disorders frequently require medical interventions such as feeding tubes, catheters, or extended hospital stays, which create additional avenues for bacterial entry into the body. (Garcia et al., 2019) In comparison, neurotypical children may not need that level of healthcare or medical interventions and thus are less exposed to bacterial entry. The frequent exposure to healthcare environments and medical devices can also expose children with neurodevelopmental disorders to antibiotic-resistant bacteria which can explain the major disparity in the resistance pattern. Children with neurodevelopmental disorders often undergo repeated hospitalizations and medical procedures, which increase their risk of being colonized by multidrug-resistant organisms (Jones & Miller, 2022).

In conclusion, the disproportionate number of bacterial isolates and the prevalence of Staphylococcus species as well as their vulnerability to bacterial infections due to their significantly higher rates of antibiotic resistance in children with neurodevelopmental disorders might be caused by a combination of immune system dysregulation, behavioral factors, and increased healthcare exposure. These combined aspects explain the pronounced difference in bacterial presence between neurotypical individuals and children with neurodevelopmental disorders, revealing a need for specialized approaches or alternative treatment strategies to manage infections in this vulnerable population (Smith et al., 2023).

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