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ISOLATION AND CHARACTERIZATION OF GRAM-NEGATIVE (*E.coli)* MULTIDRUG RESISTANCE BACTERIA FROM POULTRY FEED AND POULTRY WATER

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Abstract : Multidrug resistance (MDR) in bacteria develops the antimicrobial resistance (AMR) against the action of antibiotics. AMR mostly takes place in gram-negative bacteria which is highly recessive due to its thick peptidoglycan outer membrane layer. Gram-negative bacteria, such as *Escherichia coli*, are characterized by their unique cell wall structure, In poultry, a variety of bacterial strains can be found, including *Salmonella, Campylobacter,* and different strains of *E.coli*. These bacteria are often associated with foodborne illnesses and are closely monitored in the food industry. Among them, *E.coli* can be isolated and characterized based on its biochemical properties and specific structural features, making it an important pathogen to study in poultry-sourced bacteria.

IndexTerms- Multi-Drug Resistance, Anti-microbial Resistance, Gram- negative bacteria

INTRODUCTION

1.1 Isolation Of *E.coli*

Multiple methodologies exist for the isolation of *Escherichia coli*. The selection of a specific method is contingent upon the target strain and the intended purpose of isolation. The capacity to ferment lactose facilitates the utilization of MacConkey agar, which serves to differentiate *E.coli* from other non-lactose fermenting coliforms present in fecal, stool, food, water, and soil samples. A sample suspension (for solid specimens) may be prepared at any concentration, such as 5% in peptone water, and subsequently inoculated onto MacConkey agar, followed by incubation for a duration of 18–24 hours at 37°C. Colonies that exhibit a pink coloration and possess a round, medium size are selected as suspected *E.coli* colonies. All *E.coli* strains are amenable to capture on MacConkey agar, thereby providing a comprehensive array of strains for further investigation. The incubation of the inoculated culture media at 45°C specifically promotes the selection of thermophilic *E.coli* strains.

1.2 CHARACTERIZATION METHODS

Confirmation of *Escherichia coli* isolates can be achieved through a variety of methodologies which may include biochemical, enzymatic, or molecular techniques that are designed to accurately identify the presence of this specific bacterium. The selection of the most appropriate method to utilize for confirmation purposes is contingent upon a multitude of factors, which encompass not only the availability of various resources but also considerations such as the required specificity, sensitivity, and the context in which the testing is being conducted. Among the various confirmation methodologies that are available, biochemical methods play a significant role and are exemplified by techniques such as the IMViC series of tests, gram staining procedures, and the catalase test, each of which contributes uniquely to the identification and characterization of *E.coli* isolates in a laboratory setting.

1.2.1 INDOLE TEST

The indole assay serves to ascertain the capacity of a bacterium to synthesize indole from the amino acid tryptophan. *Escherichia coli* exhibits the enzyme tryptophanase, which catalyzes the hydrolysis of tryptophan into indole, pyruvate, and ammonia. In the indole assay, bacterial cultures are cultivated in a medium that contains tryptophan. Subsequent to the incubation period, Kovac's reagent is introduced; a positive outcome is signified by the emergence of a red or pink ring at the surface of the medium, thereby validating the production of indole (Cappuccino & Sherman, 2014).

The indole assay constitutes a critical biochemical evaluation in the taxonomy of *Escherichia coli*. The manifestation of a positive indole reaction, in conjunction with additional biochemical characteristics, facilitates the differentiation of *E.coli* from other coliform bacteria, such as *Klebsiella and Enterobacter*, which are generally characterized by their indole-negative status (MacFaddin, 2000).

1.2.2 Methyl Red and Voges-Proskauer (MR-VP) Tests

The Methyl Red (MR) and Voges-Proskauer (VP) assays constitute two components of a unified examination designed to differentiate between bacterial strains that generate stable acidic by-products through mixed-acid fermentation and those that yield neutral by-products via 2,3-butanediol fermentation.

Methyl Red Test (MR): *Escherichia coli* serves as a representative of mixed-acid fermenters, indicating its capacity to synthesize various stable acids (including lactic acid, acetic acid, and formic acid) during the process of glucose fermentation. Subsequent to the incubation phase, the introduction of the methyl red indicator leads to the manifestation of a red hue, signifying a positive result attributable to the acidic pH registering below 4.4.

Voges-Proskauer Test (VP): This analytical procedure identifies the existence of acetoin, a non-acidic metabolic by-product resulting from glucose fermentation in microbial species such as *Enterobacter* and *Klebsiella*. *Escherichia coli* generally yields a negative result for the VP assay, thereby facilitating its distinction from other microorganisms that metabolize glucose, resulting in less acidic fermentation products (MacFaddin, 2000).

Scientific Significance: The Methyl Red and Voges-Proskauer tests are fundamental for the identification and differentiation of *Escherichia coli* from other coliform bacteria within the domains of clinical and environmental microbiology. The manifestation of a positive Methyl Red test alongside a negative Voges-Proskauer test is indicative of *Escherichia coli* and is vital for its accurate identification (Tortora, Funke, & Case, 2020).

1.2.3 Citrate utilization

The citrate utilization test assesses an organism's capacity to utilize citrate as a carbon source and ammonium salts as a nitrogen source. *E.coli* is usually citrate negative due to the absence of necessary transport proteins for citrate uptake. In the citrate test, bacteria are cultured on a citrate-containing medium with a pH indicator (bromothymol blue). A positive result, indicated by a blue medium, signifies citrate utilization and a pH increase from alkaline by-products (Greenberg, 1992).

The citrate utilization test is crucial for distinguishing *E.coli* (citrate negative) from citrate positive enteric bacteria like Klebsiella and Enterobacter species. This differentiation is vital for the precise identification of pathogens in both clinical and environmental contexts (Atlas, 2019).

1.2.4 Gram staining

Gram staining represents a pivotal differential staining methodology employed to categorize bacteria into two principal classifications: Gram-positive and Gram-negative, contingent upon the architecture of their respective cell walls.

Escherichia coli is classified as a Gram-negative bacterium, thus it will manifest as pink or red when observed under the microscope owing to the retention of the safranin counterstain. This phenomenon occurs because Escherichia coli possesses a relatively thin peptidoglycan layer within its cell wall, accompanied by an outer membrane that comprises lipopolysaccharides, which fail to retain the crystal violet-iodine complex after the decolorization process (Beveridge, 2001).

1.2.5 Catalase test

The catalase assay serves to identify the existence of the enzyme catalase, which catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. When bacterial strains that express catalase are subjected to H_2O_2, the rapid effervescence resulting from oxygen liberation signifies a positive outcome. *Escherichia coli* is classified as catalase positive, indicating its capability to mitigate reactive oxygen species through the breakdown of H_2O_2 (Forbes, Sahm, & Weissfeld, 2007).

1.3 *E.coli*- Gram Negative Bacteria

Escherichia coli continues to be one of the most prevalent etiological agents responsible for a variety of common bacterial infections in both humans and animals. *E.coli* is the primary causative agent of enteritis, urinary tract infections, septicemia, and other significant clinical conditions, including neonatal meningitis. Moreover, *E.coli* is significantly linked to instances of diarrhoea in both companion and agricultural animals. The effectiveness of therapeutic interventions for *E.coli* infections is jeopardized by the emergence of antimicrobial resistance. The incidence of multidrug-resistant *E.coli* strains is escalating globally, primarily attributable to the dissemination of mobile genetic elements, such as plasmids. Additionally, the proliferation of multidrug-resistant strains of *E.coli* is noted. Consequently, the dissemination of resistance in *E.coli* constitutes an escalating public health challenge.

1.4 Anti microbial resistance

Most fields of the current medicine depend on the availability of the antimicrobial drugs. Antimicrobial resistant is the natural process occurs in the microorganism when they are introduced to different anti-drugs. Any microorganism that are exposed under selective antibiotics will be resistant or susceptible based on the characteristic features of the organism. These organisms especially bacteria that are susceptible to antibiotics will be inhibited or killed thus the multiplication of specific organism is controlled, while

the bacteria which no longer respond to the antibiotics will create antibiotic resistance, hence leads to the survival and multiplication of the bacteria (Prestinaci et al., 2015). In addition, zoonosis also poses a serious transmission factor of AMR, where antibiotic-resistant bacteria are spread between animals and humans, through either direct or indirect interaction, as well as through foodborne or waterborne events (Amin et al., 2020).

1.4.1 Biological Barrier of AMR Bacteria

Bacterial infections are transmitted by various ways that contribute large impact on the human health. It is due to the AMR of the bacteria to the selective antibiotics. Bacteria are prokaryotic and ubiquitous organisms, carry their genetic information in a form of double-standard circular DNA. They have the ability divide and multiply rapidly under the suitable conditions. Based on the cell wall characteristics they are classified as Gram-negative or Gram-positive, plays an important role in the antibiotic susceptibility and resistance (Doron & Gorbach.,2008).

Most of the antibiotic susceptibility is seen in the Gram-positive bacteria due to its thick (20-80nm), porous peptidoglycan layer that easily allows the antibiotics. These antibiotics efficiently inhibit the multiplication of the bacteria by recognising the targeting site to block its activity (Josephine et al., 2020). Gram-negative bacteria are different from Gram-positive bacteria, they have an envelope layer consist of three layers.

The first layer is the outer membrane (OM), a protective and a unique feature that distinguishes Gram-negative bacteria from Gram-positive bacteria. The OM has phospholipids that are bound to the inner leaflet of the membrane, and lipopolysaccharide (LPS) bound to the outer leaflet which is known to cause endotoxic shock. Moreover, the OM contains proteins called the outer membrane proteins (OMPs) such as porins and others which allow the passage of small molecules like amino acids and small saccharides. This outer membrane layer does not allow the antibiotics inside the bacteria, impede the activity of the organisms.

The second layer is the peptidoglycan cell wall which is a rigid exoskeleton that determines the cell shape and consists of a repeat unit of the disaccharide N-acetyl glucosamine-N-acetylmuramic acid. The third layer is the inner membrane (IM) which is a phospholipid bilayer that is responsible for multifunctional processes like structure, transport, and biosynthetic functions.

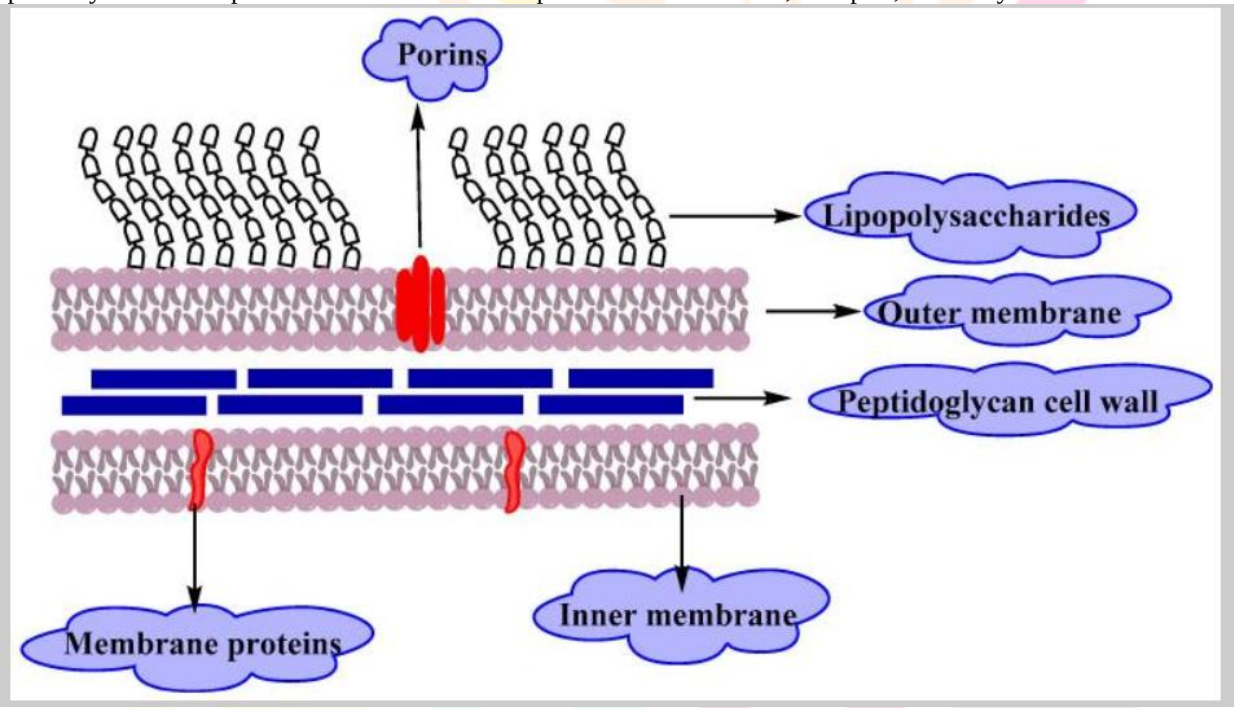

Figure 1.1.1 Cell wall structure of the Gram-negative bacteria (Breijye. et al.,2020)

1.4.2 Causes of AMR

Bacteria can develop resistance to antibacterial medicines in several ways. Some microorganisms are naturally resistant to specific antibiotics. More concerning is acquired resistance, in which bacteria that were previously susceptible to antibiotics become resistant because of exposure to these medications. This resistance can spread through a variety of mechanisms, such as genetic changes or the acquisition of resistance genes from other bacteria.

Bacterial mutations can alter the protein targeted by drugs, making them less effective. They may manufacture additional enzymes that deactivate the medication. They can modify their outer membrane proteins to prevent drugs from entering the cell. They increase the activity of transporters of the cell.

These modifications allow resistant bacteria to survive and grow while susceptible bacteria are killed by the antibiotic, a phenomenon known as vertical evolution. Bacteria can acquire resistance genes from other bacteria via horizontal evolution, which uses techniques that include conjugation, transduction, and transformation. Bacteria transfer resistance genes through direct contact, usually with a pilus. Bacteriophages (viruses that infect bacteria) transmit resistance genes between bacteria. Bacteria absorb DNA

from their surroundings, which is frequently released by deceased bacteria. These processes can cause bacteria to develop resistant to many kinds of antibiotics, which is particularly problematic in healthcare settings. This capability is due to numerous mechanisms, including alterations of cell permeability that can reduce intracellular antibiotic concentration (Li & Nikaido., 2004), antibiotic alteration, antimicrobial inactivation (Wright, G. D.,2005), modifications to antibiotic target sites (Wilson., 2014), and biofilm formation.

The global spread and diffusion of gram positive bacteria such as oxacillin-resistant Staphylococcus aureus (ORSA), vancomycin-resistant Enterococcus spp. (VRE), and Gram-negative bacilli (GNB) (including extended-spectrum-β-lactamase [ESBL] producers), carbapenem-resistant Enterobacteriaceae (CRE), and non-fermenters such as Pseudomonas aeruginosae and Acinetobacter spp. have evidenced the capability of surviving treatment with many antimicrobial agents, even the most recent ones.

Bacterial infections are a common and serious complication of cirrhosis. The most common infections are spontaneous bacterial peritonitis (SBP) and urinary tract infections. Despite diagnostic and therapeutic advances, SBP continues to cause high morbidity and mortality. Selective bowel cleansing, usually with norfloxacin, has been shown to be useful in preventing SBP. The benefit of both primary prophylaxis in patients with gastrointestinal bleeding secondary prophylaxis in patients who have recovered from a first episode of hypertension. In addition, patients with low ascitic fluid protein, especially those with low platelet count and/or or high bilirubin is a risk for SBP (Cereto, et al., 2003). However, no consensus has been reached on primary prevention in this particular group. Several studies have shown the emergence of quinolone-resistant *Escherichia coli* strains in the faecal flora. of cirrhotic, and non-cirrhotic patients receiving quinolone prophylaxis. However, the occurrence of quinolone-resistant *E.coli* SBP has been rarely reported and a possible explanation for this is thought to be the less invasive capacity of quinolone-resistant *E.coli*. Recently, quinolone-resistant gram-negative SBP. bacilli (57% appeared). to determine the prevalence of quinolone-resistant *E.coli* SBP and to determine the clinical characteristics of patients with this disease.

Enterobacter spp. are facultative, anaerobic, motile Gram-negative bacteria. There are normally commensals present in the gut of man, but they can cause infections in immunosuppressed or hospitalized subjects. Many strains exhibit numerous resistance mechanisms, such as extended-spectrum beta-lactamase, metallo-beta-lactamase and carbapenemases, which confer resistance to almost all antibiotics (Di Franco et al., 2021).

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium in the family of Enterobacteriaceae. It is generally found in the gastrointestinal tracts of poultry, humans, and other animals (Tang, et al., 2023).

1.5 Multiple Drug Resistance (MDR):

Antimicrobial drugs have undergone evolution to enhance their resistance against diverse microbial species. Resistance to multiple drugs have become increasingly prevalent globally and pose a significant threat. A microbial response that fails then, prolonged illness, and increased medical costs are the results of resistance mechanisms that are emerging and decreasing the effectiveness of treatments for common infectious diseases. With higher levels of multidrug resistance (MDR), mortality, and morbidity, most microbes, including viruses, bacteria, fungi, and parasites, have earned the unsettling moniker "Superbugs."

Insensitivity of a microorganism to antibiotics conducted or antimicrobial resistance is the simplest definition of multidrug resistance. Treatment costs have gone up because of MDR. A small number of medical procedures, such as organ transplantation and cancer chemotherapy, have helped to develop MDR.

1.5.1 Mechanisms of MDR

A common mechanism of action for antimicrobial drugs is to block the metabolic pathways of microorganisms, such as nucleotide biogenesis, which in turn blocks the synthesis of proteins, DNA/RNA, and cell envelope elements. As a result of their continued exposure to drugs, bacteria have evolved a wide range of strategies to evade the effects of drugs.

In order for bacteria to survive, their cell walls are essential. Drugs bind to the peptidoglycan layer in bacteria or interfere with the synthesis of ergosterol, as was previously discussed, inhibiting the synthesis of cell walls.These organisms experience specific chromosomal mutations or the conjugation or transformation (horizontal gene transfer) of extrachromosomal DNA elements, as in the case of *K.pneumoniae*. These mutations can alter the composition of the cell membrane (for example, by reducing the amount of ergosterol in the fungal plasma membrane), which can reduce permeability and the uptake of drugs into the cell.

Another mechanism of MDR has been identified as overexpression of drug target enzymes, which results in target bypass due to modifications in certain metabolic pathways (e.g., azoles and allylamines in fungi), resulting in the production of alternate target molecules and interference with protein synthesis. This may affect the availability of drugs at the intended locations.

MDR can also result from the inactivation or enzymatic degradation of antimicrobials by hydrolysing ester or amide bonds (such as resistance to β-lactams because of β-lactamases, etc.) and chemically transforming these compounds by acetylation, phosphorylation, adenylation, glycosylation, and hydroxylation. Genes encoding for ATP-binding cassette (ABC) transporter membrane proteins, such as P-glycoprotein (Pgp), are overexpressed. These proteins are also referred to as multidrug efflux pumps and are responsible for the export or expulsion of drugs from cells. Overexpression of these genes typically results in MDR and maintains normal cellular functions.

Cancer cells also use MDR, which restricts the use of chemotherapy over an extended period. Understanding the mechanisms underlying chemoresistance, which can arise either during therapy (innate) or at the start of therapy, reveals that the cancer cells overexpress certain multidrug resistance proteins (e.g., MRP and Pgp), which induce DNA repair mechanisms, inhibit apoptosis,

change drug targets, modify the composition of cell membranes, and promote increased drug efflux, preventing proper diffusion into the cells.

1.5.2 Interventions for MDR

MDR is rising and because of that the previously curable diseases are causing a problem and leads to death in this era. Several antimicrobial stewardship programs (ASPs) are implemented to antimicrobial therapy, reduce treatment-related cost, improve clinical outcomes and safety, and minimize or stabilize MDR.

To decrease the rise and spread of MDR, cooperative efforts are requisite because diseases which were curable earlier are becoming major causes of deaths in this era. In fact, various antimicrobial stewardship programs (ASPs) are conducted nowadays to optimize antimicrobial therapy, reduce treatment-related cost, improve clinical outcomes and safety, and minimize or stabilize MDR. ASPs Interventions are either by restricting the availability of few antimicrobial agents, called as "front-end," or by observing usage of antibiotics and then streamlining or discontinuing it, called as "back-end". Thus, there is an urgent need of support and coordination at the global, regional, subregional, and national level to serve in the future. (Tanwar, et al.,2014).

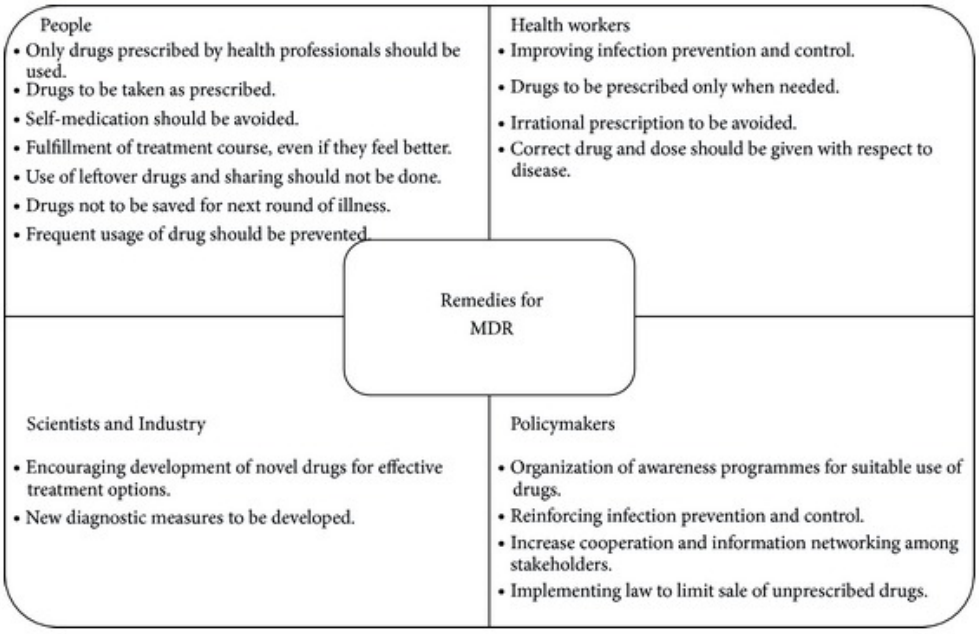

Figure 1.2.1 Interventions of MDR (Tanwar et al.,2014)

(Image courtesy: https://pubmed.ncbi.nlm.nih.gov/251)

NEED OF THE STUDY

Poultry zoonosis is the spread of diseases from poultry to people, which is often caused by zoonotic bacteria capable of developing multidrug resistance (MDR). These MDR bacteria, which can be dangerous to both humans and animals, are frequently detected in poultry facilities. Contamination can occur from a variety of sources, including chicken feed and water, which frequently come into touch with the birds' saliva, encouraging the spread of MDR bacteria. To better understand and control these infections, it is critical to isolate and characterize the organisms found in such samples. This approach aids in the identification of the exact bacteria involved, including any resistant strains, allowing for more targeted therapies in the management of zoonotic infections.

RESEARCH METHODOLOGY

The methodology section explains the approach and procedures employed to carry out the research. This part includes the collection of sample from the poultry farm, preparation of sterile media used for the growth of bacteria, serial dilutions, culturing of bacteria in media and characterization procedure of *E.coli* .

3.1 Collection of Sample from the Poultry Farm

In microbiological studies, collecting samples from the environment, such as poultry farms, is crucial for understanding microbial contamination and the prevalence of pathogens. Poultry feed and water are common vectors for bacterial transmission, particularly in intensive farming operations where hygiene may vary. It is essential to collect these samples aseptically to prevent external contamination and to ensure that the samples accurately represent the farm's microbial environment (Bhunia, 2018). During sampling, care must be taken to use sterile containers and instruments to avoid introducing contaminants that could affect the results.

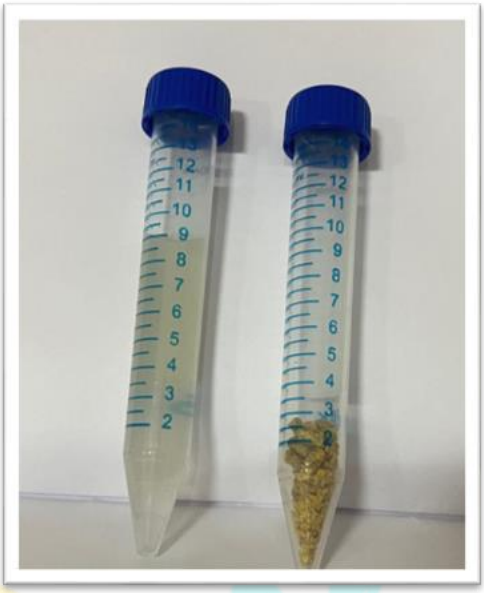

Figure: 3.1.1 Collected Sample

3.2 Preparation of Sterile Media

Various types of media is used for the luxuriant growth of the culture.

3.2.1 Preparation of Peptone Water

Peptone water is a nutrient-rich liquid medium commonly used for the cultivation of bacteria in microbiological studies. It supports the growth of a wide variety of microorganisms by providing essential nutrients like peptone, a soluble protein digest derived from animal tissues, and sodium chloride, which helps maintain osmotic balance. Preparing sterile media is critical for ensuring that only the microorganisms present in the sample are cultured, preventing any external contaminants from affecting the results (Willey, Sherwood, & Woolverton, 2017). For this experiment, peptone water is prepared at a concentration of 0.1%, meaning 0.1 grams of peptone is dissolved in 100 milliliters of distilled water to create an isotonic solution suitable for bacterial growth.

Table 3.2.1 Composition of peptone water

Ingredients	gms/L	gms/ml
Peptone	10	1
Sodium Chloride	5	0.5

0.1% peptone water is 0.1g in 100 ml of dH2O.

3.2.2 Preparation of MC Agar (MacConkey Agar)

MacConkey Agar is a selective and differential medium designed to isolate Gram-negative enteric bacteria and differentiate them based on lactose fermentation. The medium contains bile salts and crystal violet, which inhibit the growth of Gram-positive bacteria, making it selective. The presence of lactose and neutral red allows for differentiation; lactose fermenters produce acid, causing the neutral red indicator to turn pink. Non-fermenters will remain colourless. This selective property is crucial for isolating potential pathogenic enterobacteria from poultry feed and water samples, where contamination by Gram-negative bacteria such as *E.coli* is common (Atlas, 2019). The agar is prepared for 5g in 100ml.

Figure: 3.2.1 Peptone water and MC media

3.2.3 Preparation of EMB Agar

Eosin-Methylene Blue (EMB) agar is another type of selective and differential medium, particularly used for the isolation of Escherichia coli and other coliforms. The medium contains eosin and methylene blue dyes, which inhibit Gram-positive bacteria and differentiate lactose fermenters. *E.coli*, a vigorous lactose fermenter, produces dark colonies with a metallic green sheen due to the uptake of the dyes by acid production. This property allows microbiologists to differentiate *E.coli* from other coliforms and

assess their presence in samples, which is vital for understanding contamination levels in poultry water (Tortora, Funke, & Case, 2020). We have prepared agar for 3.5gms in 100ml.

Figure: 3.2.2 EMB agar

3.3 Log Dilutions

Serial dilution is a fundamental microbiological technique used to decrease a bacterial concentration to a countable level. This method involves systematically diluting a sample in a sterile diluent, often tenfold, to reduce the number of cells, which allows for accurate quantification and isolation of bacteria when plated on agar. Serial dilutions are particularly important when working with samples that contain high microbial loads, such as poultry water, to ensure the bacterial count falls within a range suitable for enumeration (Madigan et al., 2021).

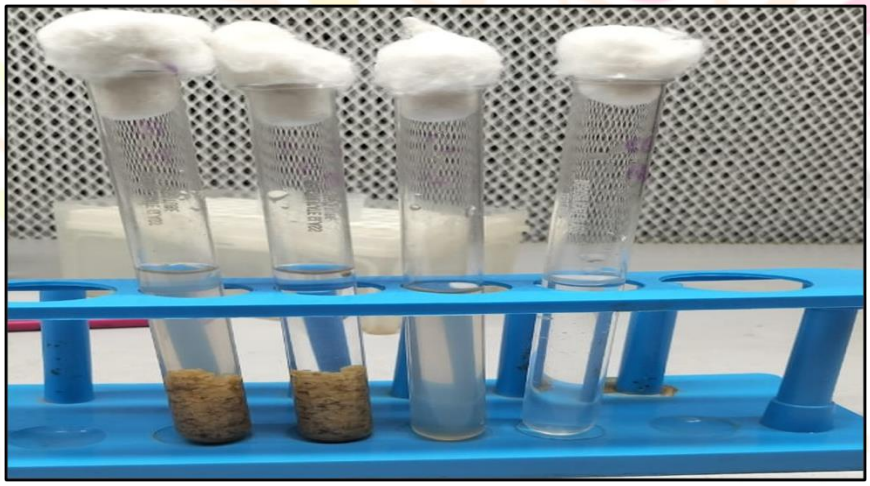

Figure: 3.3.1 Serial Dilution

3.4 Culturing of Bacteria

The pour plate technique is a common microbiological method used for isolating bacteria from mixed cultures. In this technique, a diluted microbial sample is mixed with molten agar and poured into a petri dish. As the agar solidifies, bacteria become immobilized and form distinct colonies within the medium. This method is particularly useful for obtaining pure cultures from samples containing multiple bacterial species, such as poultry feed or water. By incubating the plates at 37°C for 24 hours, optimal conditions are provided for the growth of many mesophilic bacteria, including potential pathogens like *E.coli* (Pelczar, Chan, & Krieg, 2018).

For culturing and isolating the bacteria Spread Plate Technology is used as to isolate pure culture from the mixed bacterial cultures. These plates are then incubated at 37°C for 24 hours.

3.4.1 Subculture

Subculturing is a technique used to transfer a microorganism from one culture medium to another to maintain its growth and viability or to perform further testing. In this study, *E.coli* was subcultured onto EMB agar from a diluted poultry water sample. This step is crucial because it allows for the further isolation and identification of *E.coli* based on its characteristic growth pattern on EMB agar. The metallic green sheen indicates vigorous lactose fermentation and confirms the presence of *E.coli*, differentiating it from other bacteria that may be present in the sample (Talaro & Chess, 2015).

E.coli was isolated from the poultry water in 10-2 dilution, and it was subculture on another petri plate of Eosin-methylene Blue agar (EMB agar).

EMB agar is a selective differential media for identifying the presence of *E.coli*. In presence, it produces black colonies undergoes amide bonding of the dyes in acid showing amide bonding of the dyes in acid showing metallic green sheen.

After isolating a single colony from EMB agar, the next step in microbiological analysis often involves growing the bacteria in a nutrient-rich liquid medium, such as Luria-Bertani (LB) broth. LB broth is a widely used medium in microbiology for cultivating *Escherichia coli* and other bacteria. It supports robust bacterial growth due to its balanced composition of tryptone, yeast extract, and salt (Sezonov, Joseleau-Petit, & D'Ari, 2007).

In this experiment, LB broth without sodium chloride (NaCl) is used. Omitting NaCl from the medium can alter the osmotic conditions and may be used to study bacterial responses under different stress conditions or to modify the growth environment (Sambrook & Russell, 2001). Sodium chloride is commonly included in LB broth to maintain osmotic balance, but its exclusion might be purposeful to study how *E.coli* adapts to different osmotic pressures, especially when grown in environments that mimic low-salt conditions, like some internal body tissues or freshwater environments.

3.4.2 Incubation in Shaker Incubator

The culture is incubated in a shaker incubator at a temperature of 37°C for 18-24 hours. The shaker incubator serves a critical function in bacterial culture by providing consistent temperature and aeration. The shaking motion ensures that oxygen is evenly distributed throughout the liquid medium, promoting aerobic respiration and optimal growth of *E.coli.* The temperature of 37°C is ideal for *E.coli* growth because it mimics the bacterium's natural habitat, the intestines of warm-blooded animals, where it is normally found (Atlas, 2019).

The 18–24-hour incubation period allows the bacteria to reach the log phase of growth, where cells are actively dividing and metabolically active. During this time, the bacterial population increases exponentially, making it ideal for subsequent experiments or analysis. The log phase is particularly important for experiments involving plasmid transformation, antibiotic testing, or any procedure requiring actively growing cells (Neidhardt, 1996).

By using a single colony from the EMB agar plate, researchers ensure that the culture is clonal, originating from a single bacterial cell. This is critical for consistency in experiments, as it ensures that any observed phenotypic traits or behaviours are due to the characteristics of a single strain, rather than a mixture of different bacterial strains (Pelczar, Chan, & Krieg, 2018).

This step of transferring a single *E.coli* colony from EMB agar to LB broth without sodium chloride and incubating it in a shaker incubator is a fundamental microbiological technique. It is used to cultivate a pure, actively growing bacterial culture under controlled conditions, allowing for detailed study of bacterial physiology, response to environmental changes, or preparation for further experimental procedures.

3.5 Characterization

Characterizing bacteria like *Escherichia coli* (*E.coli*) involves a series of biochemical tests that help determine their morphological and metabolic traits. These tests are critical for identifying *E.coli* and differentiating it from other similar bacteria. The following methods are commonly used for this purpose.

3.5.1 Gram staining

Gram staining is a fundamental differential staining technique used to classify bacteria into two major groups: Gram-positive and Gram-negative, based on the structure of their cell walls.

Prepare a bacterial smear on a glass slide by spreading a small amount of bacterial culture. Heat-fix the smear by passing the slide briefly through a flame. Apply crystal violet dye to the slide and let it sit for about 1 minute. Rinse with water and apply iodine solution, which acts as a mordant to form a complex with crystal violet. Wash the slide with alcohol or acetone for decolorization; this step differentiates Gram-positive from Gram-negative bacteria. Counterstain with safranin for about 1 minute, then rinse with water and blot dry.

E.coli is a Gram-negative bacterium, so it will appear pink or red under the microscope due to the retention of the safranin counterstain. This is because *E.coli* has a thin peptidoglycan layer in its cell wall and an outer membrane containing lipopolysaccharides, which do not retain the crystal violet-iodine complex after decolorization (Beveridge, 2001).

3.5.2 Indole Test

The indole test is used to determine a bacterium's ability to produce indole from the amino acid tryptophan. *E.coli* possesses the enzyme tryptophanase, which hydrolyzes tryptophan into indole, pyruvate, and ammonia. In the indole test, bacteria are grown in a medium containing tryptophan. After incubation, Kovac's reagent is added; a positive reaction is indicated by the formation of a red or pink ring at the top of the medium, confirming indole production (Cappuccino & Sherman, 2014).

The indole test is a key biochemical test in the identification of *E.coli*. A positive indole test, combined with other biochemical properties, helps distinguish *E.coli* from other coliforms like *Klebsiella* and *Enterobacter*, which are typically indole negative (MacFaddin, 2000).

Table 3.5.1 Preparation of Tryptone broth

Ingredients	gms/L	gms/ml
Tryptone broth	15	0.75

Table 3.5.2 Composition of Kovac's reagent

INGREDIENTS	gms/L	gms/ml
p-Dimethylaminobenzaldehyde	5 gms	0.5grams
Hydrochloric acid	50ml	5ml
Isoamyl alcohol	50ml	5ml

Distilled water: To make up the final volume to 1000 mL and 100ml respectively.

The indole test is a key biochemical test in the identification of *E.coli*. A positive indole test, combined with other biochemical properties, helps distinguish *E.coli* from other coliforms like *Klebsiella* and *Enterobacter*, which are typically indole negative (MacFaddin, 2000).

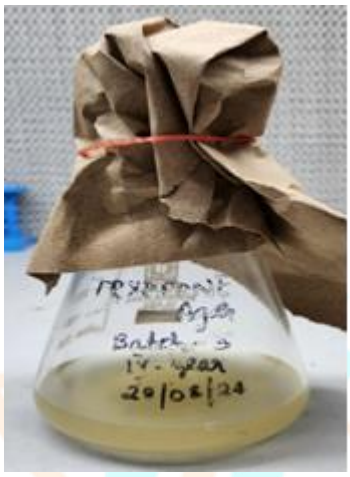

Figure: 3.5.1 Tryptone broth

3.5.3 Methyl Red and Voges-Proskauer (MR-VP) Tests

The Methyl Red (MR) and Voges-Proskauer (VP) tests are two parts of a single test used to distinguish between bacteria that produce stable acid end products via mixed-acid fermentation from those that produce neutral end products via 2,3-butanediol fermentation.

Methyl Red Test (MR): *E.coli* is a mixed-acid fermenter, meaning it produces several stable acids (such as lactic acid, acetic acid, and formic acid) during glucose fermentation. After incubation, the addition of methyl red indicator results in a red colour, indicating a positive test due to the acidic pH (below 4.4).

Table 3.5.3 Composition of MR-VP broth

INGREDIENTS	gms/L	gms/ml
Buffered peptone	7grams	0.7
Dextrose	5grams	0.5
Dipotassium phosphate	5grams	0.5

Voges-Proskauer Test (VP): This test detects the presence of acetoin, a neutral end product of glucose fermentation in bacteria like *Enterobacter* and *Klebsiella*. *E.coli* typically tests negative for the VP test, which helps differentiate it from organisms that ferment glucose with less acidic by-products (MacFaddin, 2000).

Scientific Significance: The MRVP tests are essential for identifying and differentiating *E.coli* from other coliform bacteria in clinical and environmental microbiology. A positive MR test and a negative VP test are characteristic of *E.coli* and are crucial for its identification (Tortora, Funke, & Case, 2020).

3.5.4 Citrate utilization

The citrate utilization test determines an organism's ability to use citrate as its sole carbon source and ammonium salts as its sole nitrogen source. *E.coli* is typically citrate negative because it lacks the transport proteins required to import citrate into the cell. In the citrate test, bacteria are inoculated on a medium containing citrate and a pH indicator (bromothymol blue). A positive test, which turns the medium blue, indicates the utilization of citrate and an increase in pH due to the production of alkaline by-products (Greenberg, 1992).

The citrate utilization test helps differentiate *E.coli* (citrate negative) from other enteric bacteria, such as *Klebsiella* and *Enterobacter* species, which are citrate positive. This distinction is essential for accurately identifying pathogens in clinical and environmental samples (Atlas, 2019).

Figure: 3.5.3 Simon citrate agar

3.5.5 Catalase Test

The catalase test detects the presence of the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) into water and oxygen. When bacteria that produce catalase are exposed to H_2O_2, rapid bubbling due to oxygen release indicates a positive result. *E.coli* is catalase positive, meaning it can detoxify reactive oxygen species by breaking down H_2O_2 (Forbes, Sahm, & Weissfeld, 2007).

The citrate utilization test helps differentiate *E.coli* (citrate negative) from other enteric bacteria, such as *Klebsiella* and *Enterobacter* species, which are citrate positive. This distinction is essential for accurately identifying pathogens in clinical and environmental samples (Atlas, 2019).

RESULTS AND DISCUSSION

4.1 Culturing of Bacteria

4.1.1 Subculture

The spread plate technique using MC agar was employed to isolate pure cultures from mixed bacterial samples. The growth of *E.coli* on EMB agar, evidenced by the formation of metallic green sheen colonies, confirmed its presence in the diluted poultry water samples. Subculturing these colonies further verified the identification of *E.coli* based on its distinctive growth pattern. This step highlighted the effectiveness of selective and differential media in isolating specific bacterial species from mixed cultures (Talaro & Chess, 2015).

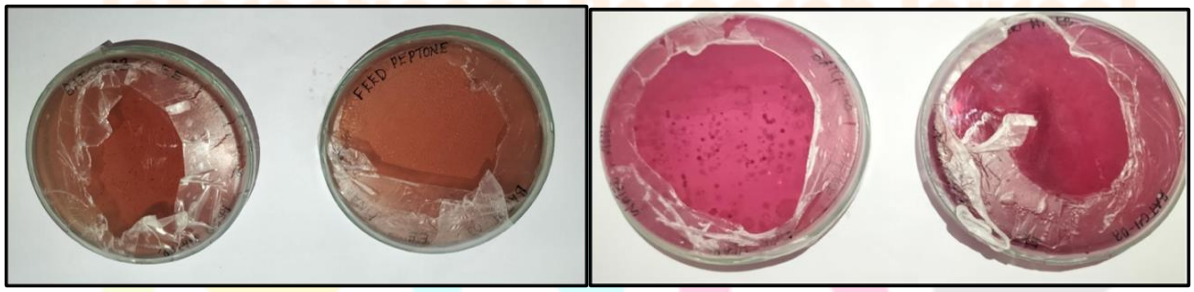

Figure: 4.1.1 Spread plate method

Figure: 4.1.2 Streaking of colony on EMB agar

4.1.2 Incubation in Shaker Incubator

After isolation, *E.coli* was incubated in Luria-Bertani (LB) broth without sodium chloride in a shaker incubator at 37°C for 18-24 hours. The shaker incubator provided optimal growth conditions through consistent temperature and aeration, promoting aerobic respiration. The bacteria reached the log phase of growth, demonstrating the effectiveness of this incubation method in cultivating a pure, actively growing culture. This step is fundamental in microbiological research for studying bacterial physiology and response to environmental changes (Atlas, 2019; Neidhardt, 1996).

Figure: 4.1.3 Growth of *E.coli* after incubation

4.2 Characterization of *Escherichia coli*

4.2.1 Gram Staining

Gram staining confirmed *E.coli* as a Gram-negative bacterium, appearing pink or red under the microscope due to its thin peptidoglycan layer and outer membrane structure. This result is consistent with the known characteristics of *E.coli* and helps differentiate it from Gram-positive bacteria (Beveridge, 2001).

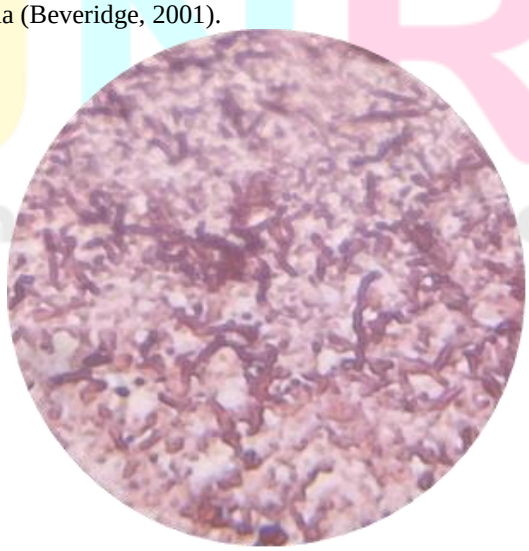

Figure: 4.2.1 Gram-staining

4.2.2 Indole Test

The indole test was positive for *E.coli*, evidenced by the formation of a red or pink ring at the top of the medium after adding Kovac's reagent. This result confirms the ability of *E.coli* to hydrolyse tryptophan into indole, further differentiating it from other coliforms that are indole-negative (Cappuccino & Sherman, 2014; MacFaddin, 2000).

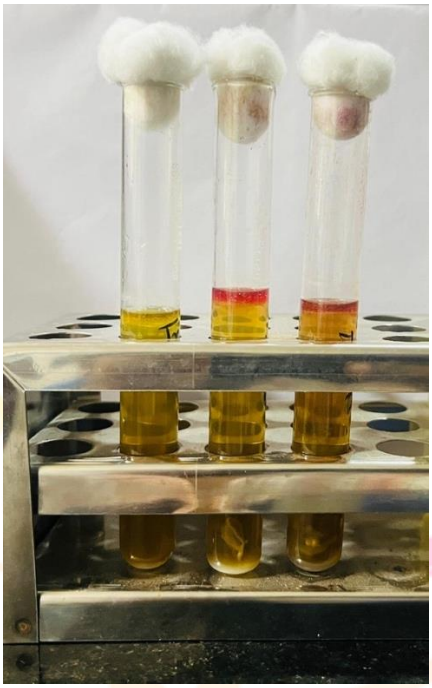

Figure: 4.2.2 Indole test

4.2.3 Methyl Red and Voges-Proskauer (MRVP) Tests

E.coli showed a positive Methyl Red (MR) test, indicated by a red color due to stable acid end-product production during glucose fermentation. The Voges-Proskauer (VP) test was negative, as expected for *E.coli*, which does not produce neutral end products like acetoin. These results are characteristic of *E.coli* and help distinguish it from other enteric bacteria such as *Enterobacter* and *Klebsiella* (MacFaddin, 2000; Tortora, Funke, & Case, 2020).

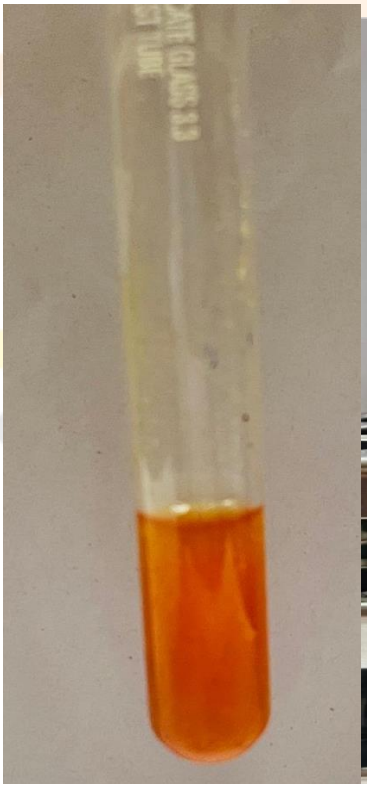

Figure: 4.2.3 Methyl Red

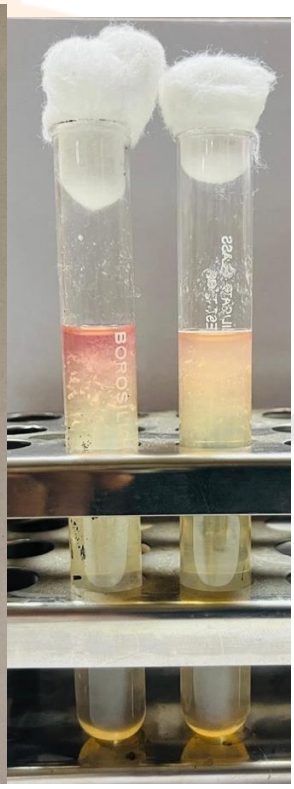

Figure: 4.2.4 Voges-Proskauer

4.2.4 Citrate Utilization Test

The citrate utilization test was negative for *E.coli*, indicating its inability to use citrate as the sole carbon source. This result is consistent with the known metabolic limitations of *E.coli*, differentiating it from citrate-positive bacteria such as *Klebsiella* and *Enterobacter*. The negative result further supports the identification of *E.coli* in clinical and environmental samples (Greenberg, 1992; Atlas, 2019).

Figure: 4.2.5 Citrate-utilization test

4.2.5 Catalase Test

The catalase test confirmed *E.coli* as catalase-positive, producing rapid bubbling when exposed to hydrogen peroxide. This result aligns with *E.coli*'s known ability to detoxify reactive oxygen species, demonstrating its oxidative stress response capability. The positive catalase test is a common feature of many aerobic and facultatively anaerobic bacteria, including *E.coli* (Forbes, Sahm, & Weissfeld, 2007).

Figure: 4.2.6 Catalase test

The results of the various biochemical tests confirm the identification of Escherichia coli from poultry farm samples. The combination of selective media, serial dilution techniques, and specific biochemical tests such as Gram staining, sugar fermentation, indole production, MR-VP tests, citrate utilization, and catalase activity provided a comprehensive characterization of *E.coli*. This study underscores the importance of using a multi-faceted approach in microbiological identification and characterization to accurately distinguish *E.coli* from other bacterial species in environmental samples.

Table 4.2.1 Characterization Of Escherichia coli

CHARATERIZATION TEST	EXPECTED RESULT	OBSERVATION	INFERENCE

Gram Staining	Gram positive – Violet colour colony Gram negative -pink or reddish colour colony	Pinkish Red colour is obtained by rod shaped cells.	Maybe *E coli*
Indole Test	Tryptone broth positive- red/violet layer Negative - yellow/slightly cloudy	Red ring was formed in test sample and no ring formation in control	Maybe *E coli*
Methyl Red	MR Positive – Red on surface MR Negative – Yellow on surface	The test sample turns red	Maybe *E coli*
Voges-Proskauer	VP Positive – Cherry red VP Negative – Yellow/Brown on surface	Cherry Red colour formation in test sample and no colour formation in control	May not be *E coli*
Citrate Utilization	Positive – Green to intense blue Negative – no colour change	Colour change from dark green to dark blue.	May not be *E coli*
Catalase	Positive – bubble formation Negative – no bubble formation	Bubble formation in test sample	Maybe *E coli*

Based on the majority of the test results, the positive Indole, Methyl Red, and Catalase tests are indicative of *E coli*. However, the positive Voges-Proskauer and Citrate utilization tests suggest that the organism may not be *E coli* or that the strain being tested has atypical results for these tests. Therefore, it is possible that this bacterium may be closely related to *E coli* or another member of the Enterobacteriaceae family, which shares some similar characteristics but differs in the VP and citrate tests. Further tests might be needed for more precise identification.

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