Comparative Analysis of Antibiotic Resistance in Bacteria From Integrated and Non Integrated Fish Farms in Bangladesh

" A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY ,NOAKHALI SCIENCE AND TECHNOLOGY UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE(MSc) IN MICROBIOLOGY"

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CERTIFICATION OF APPROVAL

This is to certify that, the research work described in this thesis entitled “Comparative Analysis of Antibiotic Resistance in Bacteria from Integrated and Non-Integrated Fish Farms in Bangladesh"conducted by Masiath Subha , is the original work and has been carried out under my supervision.l have gone through all the data/results and materials reported in this manuscript and certify their authenticity and correctness.l further certify that the material included in this thesis has not been used in part or full in a manuscript already submitted or in the process of submission in partial or complete fulfillment of the award of any other degree from other institution. I also certify that the thesis has been prepared under my supervision according to the prescribed format and I enclose its evaluation for the award of Master of Science (M. Sc.) degree through the official procedures of the Department of Microbiology, Noakhali Science and Technology University, Bangladesh.

……..

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DECLARATION

I do hereby declare that, the entire work presented as a thesis entitled " Comparative Analysis of Antibiotic Resistance in Bacteria from Integrated and Non-Integrated Fish Farms in Bangladesh" has been submitted in partial fulfilment of the requirements of the degree of Master of Science (M.Sc.) in Microbiology from Noakhali Science and Technology University.This work has been carried out in the Laboratory of the Microbiology Department,Noakhali Science and Technology University,Noakhali, Bangladesh .Without this thesis, the degree of Master of Science (M. Sc) in Microbiology will remain incomplete.

Furthermore, I declare that this work has not been submitted for any degree or diploma to another university or other institution.

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Short Form	Full Form
LB	Luria-Bertani Agar
Mac	MacConkey Agar
XLD	Xylose Lysine Deoxycholate Agar
SS	Shigella Salmonella Agar
HGT	Horizontal Gene Transfer
AMR	Antimicrobial Resistance
+ve	Positive
-ve	Negative
spp	Species
No	Number
MIU	Motility Indole Urea
TSI	Triple Sugar Iron
MHA	Mueller Hinton Agar
PCR	Polymerase Chain Reaction
NCBI	National Center for Biotechnology Informations
16S rRNA	16(Svedberg unit) Ribosomal Ribonucleic Acid
R	Resistant
S	Sensitive
I	Intermediate

Abstract

In Bangladesh,Farmers employ both integrated systems, where fish are raised alongside crops or livestock and non-integrated systems that concentrate solely on fish farming. Despite the potential dangers there is a lack of understanding regarding how antibiotic resistance differs between integrated and non-integrated fish farming systems in Bangladesh.This current study aims to compare bacterial resistance trends in these two types of farming systems, providing insights to encourage safer and more sustainable aquaculture practices.Twenty-four different Thai Pangas samples were collected from four integrated and four non-integrated fish farms across the Noakhali region, Bangladesh.The gut samples from each fish were collected and analyzed using standard microbiological techniques.Bacterial strains were identified through morphological, biochemical, and molecular methods.The isolates were then tested for antibiotic susceptibility against 15 different antibiotics using the Kirby-Bauer disk diffusion method. Furthermore, ESBLs, quinolone and tetracycline resistance genes were identified by using specific primers.Potential resistant isolates were selected and confirmed using 16S rRNA sequencing and phylogenetic analysis.A total of 90 isolates were identified from both integrated and non-integrated farms.The rate of antibiotic resistance was found to be 65% in integrated farms compared to 40 % in non-integrated farms. Among all isolates, E. coli exhibited the highest resistance rate at 22%,followed by Salmonella spp. at 19.50% and Proteus spp. at 15.8% in integrated farms.Conversely, non-integrated farms showed the highest resistance rates in E. coli (10%) and Klebsiella spp. (8%), and Proteus spp. (5%). Furthermore, major resistance genes such as *tetA, blaCTX-M ,blaTEM, sul1* and *qnr* were detected in 47%, 35%, 23%,35% and 29% of isolates from integrated farms, respectively, while the corresponding percentages in non-integrated farms were 25%, 23%, 15% and 17%.In this finding, a greater occurrence of antibiotic resistance was noted in integrated farms than in non-integrated farms. Overall, the results will aid in the development of effective aquaculture practices (EAP) designed to reduce antimicrobial resistance in aquatic environment.

Keywords: Biochemical,molecular,ESBL,antibiotic-resistance,aquaculture,phylogenetic

Chapter -1: Introduction ,Research Gap, Research Objectives

Comparative Analysis of Antibiotic Resistance in Bacteria From Integrated and Non Integrated Fish Farms in Bangladesh

1.1.1 General Introduction

The fisheries sector of Bangladesh is contributing significantly in food and nutrition security through consistently providing safer and good quality animal protein, which eventually contributes to achieve the set targets of SDGs by 2030. According to Department of Fisheries report, Bangladesh already became one of the world's leading fish producing countries with a total production of 4.76 million MT in 2023 [5]. Moreover, fisheries being economically more remunerative, have been identified as the major means of rural employment, poverty alleviation and foreign exchange earnings. Aquaculture, notably fish farming in ponds, has contributed substantially to meet the protein demand of the populations while creating employment opportunities. Fish farmers are also motivated to introduce integrated farming system such as fish-cum-poultry culture to reduce feed cost and to get more profit. An Integrated Farming System (IFS) is a sustainable agricultural approach that integrates various components such as crops, livestock, poultry, fish, and trees into a single, interdependent system.It is based on the idea of resource recycling, in which waste from one component is transformed into a useful input for another [7].By making the most use of the resources at hand, this approach increases production and income, which is especially advantageous for small-scale and marginal farmers [1].Poultry farming is commonly combined with fish farming for its cost-effectiveness.Poultry droppings act as natural fertilizers in fish ponds, promoting the growth of plankton that fish feed on.This reduces reliance on commercial feed and fertilizers, cutting overall costs by as much as 60% [2].In Bangladesh, the predominant methods of producing carps for freshwater aquaculture are semi-intensive and extensive pond polyculture. A questionnaire was created and pre-tested for 80% of the overall production of freshwater aquaculture [4–6]. Pangus, tilapia, small indigenous species (SIS) of fish, and ricefish farming account for the remaining 20% of the total [5].Fish farming is thought to employ 138.68 lakh people in Bangladesh, according to estimates [3].

Pangasius hypophthalmus, popularly known as 'thai pangas', was introduced to Bangladesh from Thailand in 1990 and has become a popular fish species like other South Asian countries due to its rapid growth rate, low mortality and year-round production [3[.Farming of Thai pangas is a significant component of aquaculture in Bangladesh, and in 2016, total

production was 494,357 tons, accounting for 29% of the total farmed fish supply in the country (Department of Fisheries Bangladesh, 2017). Considering the great potential of pangas farming and its vital role in national food security, its production should be increased to ensure good quality[13].Some different species of pangas fish found in Bangladesh include:1.*Pangasius pangasius, 2.Pangasius hypophthalmus*.These species are part of the pangasiid catfish family and are commonly found in freshwater and brackish waters in Bangladesh [14].The production diversity of Pangas fish in Bangladesh has significantly increased over the past few decades. According to the search results, the annual production of cultured pangasius has increased from 155,000 tonnes in 2010-11 to 395,000 tonnes in the 2021-22 fiscal year.The availability of juveniles, the rise in the use of commercially prepared feed, and market demand are some of the reasons for this expansion [15]. One of the most important fish species in Bangladesh's aquaculture industry, pangasius accounts for about 13% of the country's overall aquaculture output.Because of its quick growth, ability to withstand low water quality and strong market demand, the species is well-liked by both producers and consumers. Additionally, farmers have become interested in the introduction of white pangasius from Vietnam due to the potential for exporting to meet demand in other nations.[16]

The quick development of resistance among dangerous bacterial species is especially concerning since it presents a major risk to public health through exposure to the environment and the food chain.Our current study intends to explore the prevalence and genomic traits of MDR pathogenic bacteria in Thai Pangas (*Pangasius hypophthalmus*) raised in integrated and non-integrated poultry fish farming systems in Noakhali region in Bangladesh for the purpose of addressing this crucial issue.We identify and evaluate these bacterial species' phenotypic and genotypic features using modern molecular techniques such as 16S rRNA sequencing.The study provides information about the presence of important resistance genes and their potential for horizontal transfer.Furthermore, the findings of this study will contribute to a better understanding of the role of antibiotics in fish farming, emphasizing the importance of improved farming methods and responsible antibiotic usage. Raising awareness among stakeholders, including as feed dealers and veterinary practitioners, could be critical in decreasing antibiotic overuse and preventing the emergence of antimicrobial resistance in integrated aquaculture systems.

1.1.2 Research Gap

Despite the rapid expansion of aquaculture in Bangladesh, particularly the intensive farming of Thai Pangas (*Pangasius hypophthalmus*), there is a critical lack of comprehensive studies addressing the prevalence, characterization, and molecular profiling of multidrug-resistant (MDR) pathogenic bacteria in farmed cultured fish species [7].

Most existing research in Bangladesh has primarily focused on general bacterial pathogens and phenotypic antibiotic resistance profiles through the food chain and aquatic environment.Previous studies in Mymensingh and Gazipur reported predominant resistance to ampicillin and tetracycline, and high prevalence of *E. coli, Aeromonas*, and other enteric pathogens in farm cultured pangas,however they were unable to find out the antibiotic susceptibility of other third generation antibiotics including ceftriaxone, cefotaxime, ceftazidime, cefixime, cefdinir, and cefpodoxime [12].

Moreover, one study conducted in Noakhali to observe the drug-resistant bacteria in aqua cultured Pangas via phenotypic tests ,but there is a lack of advanced molecular analysis (e.g., PCR, sequencing) to detect specific resistance genes (such as *bla, qnr, tet*), plasmid/super-integron presence, or virulence factors [19]We all know biofilm (a cluster of bacteria) formation enhances persistence and resistance mechanism of bacteria, but in Bangladesh, only some aquaculture species like *Ompok pabda* have been investigated for biofilm phenotypes and genetic traits [9]. No research have been applied similar molecular-biochemical analysis to Thai Pangas,leaving a gap in understanding the actual pathogenic potential and environmental survival strategies of isolates.

On the other hand,Retail studies in metropolitan areas (e.g., Dhaka, Gazipur) have been highlighted multidrug resistant *E. coli, Salmonella*, and others in pangas, posing food safety risks.However, the farm-origin of those multidrug resistant strains are unexplored and which genes are responsible still undetected by genome analysis [11.

1.1.3. Research Objectives

The main objective of the present study is to investigate the prevalence and genomic characteristics of MDR pathogenic bacteria in Thai Pangas (*Pangasius hypophthalmus)* cultivated in integrated poultry–fish farming systems and non integrated systems in the Noakhali region of Bangladesh.

The specific objectives are as follows:

1.Isolation and comparative analysis of multidrug-resistant and pathogenic bacterial species from Thai Pangas (*Pangasius hypophthalmus*) presented in both integrated and non - integrated aquaculture systems.

2. Genomic identification of pathogenic bacteria by 16SrRNA sequencing method for characterization of antimicrobial resistance patterns.

3.Molecular identification of resistance genes, including ESBL (Extended Spectrum Beta-Lactamase), carbapenem, colistin, tetracycline resistance genes through PCR.

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Chapter-2: Literature Review

2.Literature Review

Aquaculture basically involves the cultivation of fish and other aquatic species, has emerged as a crucial as well as fast-growing sector of food production in Bangladesh.It is crucial for ensuring food and nutrition security,providing employment facilities and enhancing economic development in rural regions [7]. Because of its huge ponds, rivers, and floodplains, Bangladesh is often known as a water-rich country which is ideal place for fish cultivation.Over recent years, aquaculture has grown from a traditional, small-scale practice into a large commercial operation that benefits millions of people [1].As reported by the Department of Fisheries (DoF, 2022), Bangladesh ranks among the leading fish-producing nations globally [9].

Figure 2.1: Fish Farming in Bangladesh

More than 56% of the nation's fish consumption comes from this industry, providing a suitable portion of the population access to reasonably priced protein. It also generates remarkable revenue and employment facilities directly or indirectly supporting 18 million people particularly in rural areas [9]. Beyond its economic importance, fish farming has

cultural and culinary importance because fish is an essential part of the Bangladeshi diet, which is known as the expression "Machh-e Bhate Bangali" ("a Bengali is made of fish and rice").

2.1 Types of Aquaculture System : There are two main types of aquaculture systems in Bangladesh: integrated and non-integrated. Each has its own way of managing, interacting pathways with the environment and affecting public health, sustainability as well.

2.1.1 Integrated Fish Farming Systems

A technique known as "integrated fish farming" combines fish farming with other aspects of farming, such as raising cattle or growing crops,livestocks all inside a unified agricultural system [5].Fish with chickens, ducks, and fish with crops are typical instances of this system .In these processes,fish ponds are usually fertilized naturally with manure from livestock or crop wastes to promote the growth of plankton, an essential food source for fish and aquatic environment [5,6].Because of this nutrient recycling mechanism, integrated farming is both economically and environmentally feasible.Research has depicted that integrated systems can increase overall productivity, reduce production costs and support small-scale farming's sustainability.This approach is especially well suitable for the resource-constrained rural environment where farmers strive to maximize results from few water and land resources [10].

Figure 2.2: Different Integrated Farms in Noakhali

However, adding untreated livestock excrement directly to fish ponds can bring a variety of microorganisms including possible diseases. Antibiotic-resistant bacteria and leftover residues can contaminate the aquaculture environment when cattle are given antibiotics to prevent sickness and promote growth [2].Additionally, this could make an environment that encourages the growth of bacteria resistant to antibiotics, endangering the health of fish, farmers as well as customers [9,10].

2.1.2 Non-Integrated Fish Farming Systems

Non-integrated systems, on the other hand, are only concerned with fish production and have no connection to the production of crops and animals [12].Depending on the amount of external inputs (such feed, fertilizer, and labor) are required, these systems can be extensive, semi-intensive and intensive [11–12].While semi-intensive systems combine natural food sources with supplemental feeding and intensive systems heavily rely on commercial feeds, aeration, and water exchange for high stocking densities and rapid growth, extensive systems rely primarily on the natural productivity of ponds and require minimal inputs [11].Non-integrated systems provide farmers more control environment over disease prevention and biosecurity, however they usually depend more on commercial feeds, prolbiotics, and antibiotics to maintain the betterment of fish health [12].In addition to causing chemical residues to build up in the pond ecology, this interdependence can also encourage the evolution of resistant bacterial strains if the process is abused [12,13].

Figure 2.3: Non Integrated Farms

2.2 Mechanisms and Development of Antibiotic Resistance in Aquatic Bacteria

Aquaculture habitats are becoming regarded as significant reservoirs for resistant bacteria and gene is making antibiotic resistance one of the most alarming worldwide public health issues of the twenty-first century [22].Because of the random use of antibiotics in fish farming and the continuous interactions between aquatic animal and human microbial communities, resistance can arise, persist, and spread through a complex network.Therefore, it is pivotal to comprehend the basic mechanisms of antibiotic resistance in order to know how aquaculture contributes to this global issue [19–22].

2.2.1 Overview of Antibiotic Resistance

When bacteria acquire the ability to endure or proliferate in the presence of antibiotic concentrations that would typically prevent or eradicate them, then antibiotic resistance develops.This resistance arises naturally in aquaculture systems as a response to the selection pressure exerted by ongoing exposure to antibiotics, whether through direct pond treatments, medicated feed, or leftovers from livestock manure in integrated systems [25].When antibiotics are added to fish ponds, microbial communities are enforced to selective pressure, at that time vulnerable bacteria die while those with resistance characteristics survive and grow. These resistant strains then regarded as the predominant members of the bacterial community and the genetic components responsible for this resistance might be transferred to other bacteria within the same system [29].This pay the way to aquaculture ecosystems remarkable breeding grounds for antibiotic resistance specially when antibiotic usage is very random and inadequately regulated.

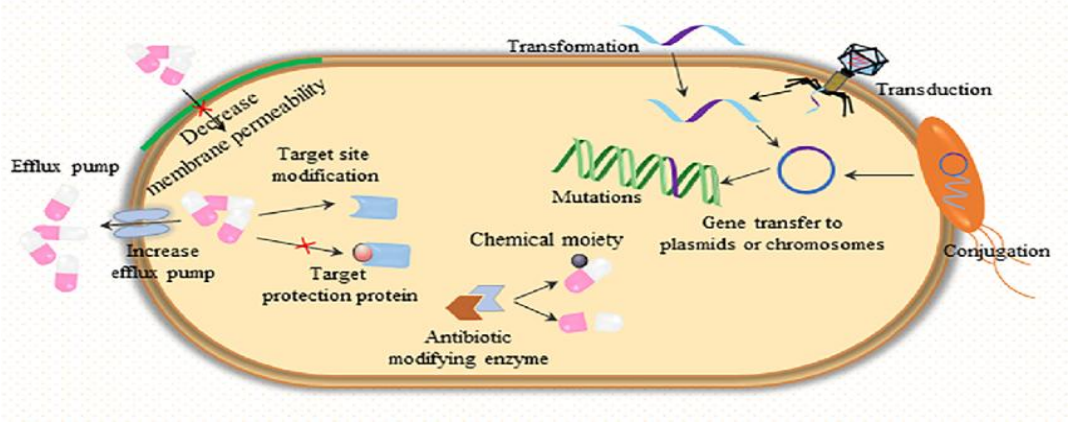


Figure 2.4: Common Mechanism of Antibiotic Resistance in Bacteria [21]

2.2.2 Genetical Mechanism of Resistance

Bacteria have evolved remarkable genetic defense mechanisms to withstand antibiotic-contaminated environments.These microbes are active and sensitive and able to alter their genetic makeup to fend off chemical assaults rather than merely giving in to antimicrobial agents [21]. Bacteria typically develop resistance via two vital genetic pathways:

1.**Mutations in chromosomal genes:**Spontaneous mutations, which are random changes in the genetic material of bacteria that occur during cell division are a simple but very effective way for bacteria to evolve resistance [24].For example, the structure and function of the target site where an antibiotic normally binds can be altered by a single mutation in a bacterial gene.In these situations, the medication is ineffective because it cannot attach efficiently and properly.Genes that produce ribosomal proteins or enzymes that are targeted by antibiotic for example those affected by tetracyclines and fluoroquinolones are often the sites of these mutations [25,26].

2.**The acquisition of resistance genes through horizontal gene transfer (HGT)**:These alteration of bacteria have a significant survival advantage in ponds, where antibiotics may remain in water or sediments for long periods of time [25].Resistant mutants flourish and proliferate while susceptible germs perish, eventually taking control of the bacterial population [26].This mechanism, known as selective enrichment, guarantees that resistance traits become long-lasting features of the microbial community.Three main HGT modes exist:

Conjugation:This is perhaps the most common way to transmit genes.A tiny circular DNA structure called plasmid is transferred from one bacterial cell to another during the time of conjugation, which occurs when two bacterial cells come into intimate contact.Multi-drug resistance (MDR) is the term for the phenomena when recipient bacteria become resistant to numerous drugs since these plasmids often carry multiple resistance genes [27, 28].

Transformation:Some bacteria have the ability to take up free-floating DNA fragments from their environment. The bacteria can incorporate these fragments into their own genetic material if they include genes that confer resistance to antibiotics.Transformation can happen often in aquaculture ponds when decomposing organic waste and lysed bacterial cells release DNA into the water [30].

Transduction:In this instance, bacteriophages that target particular bacteria function as genetic messengers. When a phage infects a bacterium, it may unintentionally encapsulate resistance genes and other parts of the cell's DNA, which it then transfers to another bacteria during a subsequent infection [29, 30].

2.2.3 Biochemical Mechanisms of Resistance

While genetic changes depict how bacteria become resistant, metabolic processes within the cell play a vital role in the bacteria's real survival during antibiotic exposure [28].Bacteria are active substances at the microscopic level and they have evolved a sophisticated defense system that allows them to destroy, avoid and expel antibiotics.These techniques are essential to their survival in antimicrobial-rich environments such as fish ponds where antibiotics are often used.Enzymatic degradation, target site modification, efflux pumps, and reduced membrane permeability are the leading biochemical processes [32].

1.**Enzymatic Degradation of Antibiotics**:The synthesis of enzymes that can chemically neutralize antibiotics is one of the most powerful resistance mechanisms employed by bacteria.These enzymes alter antibiotic molecules before they cause any harm, acting as molecular "scissors" [36].One prominent example is the synthesis of β-lactamases which are enzymes that break down the β-lactam ring, a crucial structural component of antibiotics such as cephalosporins and penicillins.The antibiotic becomes ineffective after this ring is broken because it cannot attach to its target site in the bacteria [39, 40].Many bacterial species have evolved throughout the time to produce different types of β-lactamases, some of which can even degrade more recent, sophisticated antibiotics designed to withstand

these enzymes.Other enzymes, including aminoglycoside-modifying enzymes, work by adding particular chemical groups to the antibiotic molecule, such as phosphate, acetyl, or adenyl, changing its structure and preventing it from binding to bacterial ribosomes [40].Because of its biochemical adaptability, enzymatic degradation is one of the most dangerous and common forms of resistance, especially in aquaculture environments where antibiotics are frequently used [38–40].

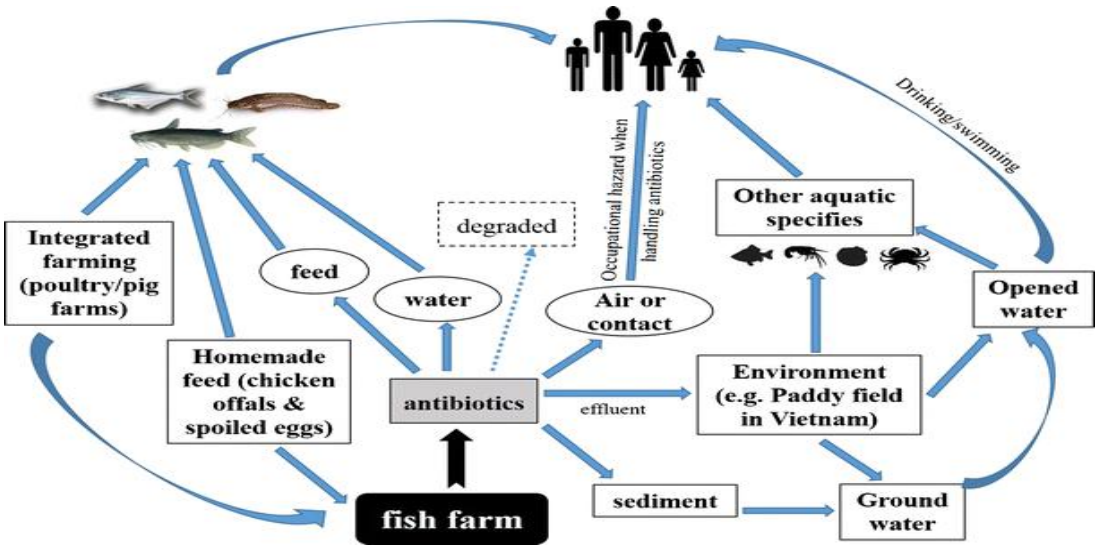
2.**Modification of Antibiotic Target Areas**:Altering the target regions inside bacterial cells where drugs normally bind is another common strategy for resistance. In order to interfere with vital cellular processes, the majority of antibiotics are designed to attach to certain molecular structures such ribosomal RNA, enzymes, or proteins involved in cell wall formation [37, 38].Nevertheless, resistant bacteria can alter these target regions by chemical changes or mutations, making it impossible for the antibiotic to recognize or bind to them [38].For instance, bacteria may change specific features of ribosomal RNA, reducing the efficacy of macrolides and tetracyclines, or they may change the DNA gyrase enzyme, which is the target of fluoroquinolones.Even in the presence of high antibiotic doses, these minute structural changes allow the bacteria to carry out their regular functions [36, 37]. In essence, the antibiotic loses its perfect "lock-and-key" match because it is unable to attach to its target, allowing the bacterium to live unharmed.

3.**Efflux Transporters:**.Another remarkable defense mechanism in bacteria is represented by efflux transporters.These particular transport proteins serve as tiny molecular pumps and are incorporated into the bacterial cell membrane [33].Their function is to actively remove dangerous materials, such as antibiotics, from the interior of the cell before the medications may accumulate to dangerous concentrations.These pumps are frequently produced in greater quantities in resistant bacteria, allowing them to function more quickly and effectively than in non-resistant cells [33, 34].As a result, even if an antibiotic manages to enter the cell, it is quickly ejected once more, much like bailing water out of a leaky boat before it can fill.Efflux pumps can be broad-spectrum (removing many different kinds of medicines at once) or very selective (targeting a specific antibiotic) [35].The latter kind makes treating infections more difficult by greatly contributing to multi-drug resistance (MDR).Efflux pumps can give bacteria a significant survival advantage and promote the maintenance of resistance strains in pond environments in aquaculture, where bacteria regularly come into contact with different antibiotics [30,31].

4.**Decreased Cell Membrane Permeability**:This mechanism proves particularly effective when combined with others such as efflux pumps or enzymatic breakdown. Together, they create a “double shield” fewer antibiotics can penetrate the cell, and those that manage to do so are quickly expelled or destroyed. This synergistic defense strategy is a key reason why Gram-negative bacteria, like *Pseudomonas* and *Aeromonas* species commonly found in aquaculture, are notoriously challenging to eradicate [27-29].For an antibiotic to be effective, it must first reach the bacterial cell. However, some bacteria, particularly Gram-negative ones, have an outer membrane that acts as a selection barrier. By changing the number or form of porin channels—tiny protein pores that allow molecules to flow through—these bacteria can decrease the permeability of their cell membrane and stop antibiotics from entering in sufficient amounts [27].

5.**Coexistence of Various Mechanisms**:Resistant in bacteria typically don't depend just on one of these methods.Rather than they often use several defense tactics at once which makes them incredibly resilient [25]. For example, a single strain of bacteria may simultaneously manufacture β-lactamase enzymes, alter its ribosomal RNA and use efflux pumps.Multi-drug resistance (MDR), a growing issue in both clinical medicine and aquaculture is exacerbated by these multilayer defenses [25, 26].Antibiotic resistance in aquaculture spreads broadly through complex environmental routes that connect aquatic systems, soils, animals, and ultimately humans; it is not confined to fish ponds or hatcheries.Evaluating the wider ecological and public health risks associated with aquaculture activities requires an understanding of how resistance genes and resistant bacteria move through these interconnected habitats [26].

2.3. Environmental Transmission Pathways of Antibiotic Resistance in Aquaculture



2.3.1 Water as a Primary Conduit:

The primary medium for the spread of resistant bacteria and antibiotic residues is water.Ponds are connected to canals, rivers, or floodplains that interact with home and agricultural systems in many fish farms, especially in Bangladesh and other low-lying tropical locations [12].These untreated effluents of ponds are frequently dumped into natural waterways, where they carry resistant bacteria, mobile genetic elements like integrons and plasmids, and leftover antibiotics [11].These bacteria can readily interact with local aquatic microorganisms after being put into open water, encouraging horizontal gene transfer (HGT).As a result, resistance genes can quickly proliferate throughout a wide range of bacterial species, including human-harming ones. Numerous investigations have found common resistance genes (such as tet, sul, and bla) in nearby rivers and aquaculture ponds, suggesting that water exchange plays a major role in genetic transmission and environmental contamination [12].

2.3.2. Sediments as Reservoirs of Resistance:

Pond sediments act as long-term storage places for resistant bacteria and antibiotic residues, while water acts as a transport medium.Sulfonamides and oxytetracycline are two examples of antibiotics that can stick firmly to sediment particles and remain there for weeks or even months [17].Even after the use of antibiotics has ceased, these residues still put selective pressure on the bacterial populations living in the sediment, encouraging the survival and expansion of resistant strains.Additionally, abundant organic matter and high bacterial populations found in sediments create the perfect milieu for horizontal gene transfer [17,18].During pond cleaning, harvesting, or water exchange, resistant bacteria from these

strata may re-enter the water column, thereby refilling the aquatic environment with resistant populations [18,19].

2.3.3. Fish and Other Aquatic Organisms as Vectors:

Antibiotic-resistant bacteria can be carried and amplified by fish and other aquatic creatures.Their stomach, skin, gills and mucus may be home to these resistant bacteria which present chances for transmission both within and between species [5]. These bacteria can spread to other aquatic systems and even to people who handle fish when it is sold or transported to markets and processing facilities [4,5].The risk of cross-contamination is greatly increased in integrated aquaculture systems, which combine fish farming with the production of livestock or crops.When manure is used as pond fertilizer, it can contaminate the aquatic ecology with resistant bacteria and drug residues from livestock [7].On the other hand, a cycle of environmental transmission linking aquaculture, agriculture, and human habitats may result from the reuse of pond water that is rich in resistant bacteria for crop irrigation

2. 3.4 . Human and Farm Management Practices

Human actions and farm management choices play a major role in determining how resistance spreads. Antibiotics are widely utilized without proper veterinary oversight in many small-scale or semi-intensive fish farms [13].Antibiotics may be mixed carelessly with feed or given by farmers as growth boosters, leaving excess residues in the water. Environmental contamination is also caused by frequent water exchanges between ponds, improper disposal of outdated antibiotics, and a lack of wastewater treatment facilities [2].Resistant bacteria may also be encountered by workers who come into touch with fish, feed, or pond water. Aquaculture workers' hands and clothing have been reported to have resistant Vibrio and Aeromonas organisms [10,11].This situation both poses health risks to workers and permits the transfer of resistant strains into household environments via contact or food handling.

2.3.5.Linkages Beyond the Farm: The One Health Perspective

The One Health concept, which recognizes the interdependence of human, animal, and environmental health is highlighted by the environmental development of antibiotic resistance in aquaculture [25, 26].Humans can contract resistant bacteria and genes from fish ponds through a variety of routes, including contaminated water, food, direct touch, or

exposure to the environment [11]. For instance, resistant bacteria may colonize human microbiota or transfer resistance genes to pathogenic species when pond effluents are dumped into rivers used for drinking or bathing.The lines separating aquaculture, agriculture, and human health get blurred in this integrated framework [11,12].It highlights the critical need for integrated efforts in antibiotic stewardship, waste management, and the monitoring of antimicrobial resistance (AMR) across all sectors because what begins as a localized farming practice can grow into a regional or even global health hazard [15].

2.4. Fish as the major concern of Gram-negative bacteria:

Fish, particularly Tilapia and Pangas are a major concern for Gram-negative bacteria due to their susceptibility to infections caused by these bacteria [21].Gram-negative bacteria are a diverse group of microorganisms that can cause a range of diseases in fish, including bacterial gill disease, fin rot, and enteric septicemia. These bacteria are of particular concern in aquaculture, where they can lead to significant economic losses due to reduced growth rates, increased mortality, and decreased market value of the fish[17]. Gram-negative organisms are a diverse group of bacteria that can be found in tilapia fish. Some of the most common gram-negative bacteria associated with fishes include:

2.4.1The Enterobacteriaceae

Gram-negative bacteria and several symbionts, including many of the more wellknown pathogens like *Salmonella, Escherichia coli, Yersinia pestis, Klebsiella* and *Shigella*, are all members of the broad family Enterobacteriaceae.This family of bacteria also includes *Proteus, Citrobacter, Serratia,* and *Enterobacter*, which can cause sickness [19].Several peptidoglycan-less insect endosymbionts within the enterobacteriales form a sister clade to the Enterobacteriaceae based on phylogenetic analysis; however, because these species lack valid descriptions, the group is not recognized as a taxon. Examples of these species include Sodalis, Buchnera, Baumannia, and Blochmannia, but not formers rickettisias [17].

2.4.2 *Escherichia coli*

A gram-negative bacteria called *Escherichia coli* is present in the lower intestine of warm-blooded mammals. Although there is a wide range of E. coli, the most of them are not contagious.They pose no threat. Microbes like *E. Col*i are also opportunistic. Intestinal

infections can be caused by specific strains of *Escherichia coli*, including 0157:H7 [20].Certain additional strains have the potential to induce fatal acute renal failure and bloody diarrhea [21]. Foods or water that are already tainted with E. Coli can infect both humans and animals [14].

2.4.3 *Pseudomonas spp*

They are enteric bacilli and vibrios morphologically.Their peritrichous flagella make them a motile species of bacteria.*Pseudomonas spp.* are widely distributed in soil, water, and nearly all man-made settings [22]. Cystic fibrosis, urinary tract infections, and other external and interior human body conditions can be caused by *Pseudomonas aeruginosa*.Additionally opportunistic microorganisms are Pseudomonas spp,Although,not each time they infect someone [21,22].

2.4.4 *Salmonella spp*

Since they can enter, multiply, and survive in human host cells, almost all strains of *Salmonella* are harmful and can cause potentially fatal illnesses [23].Because *Salmonella* is a zoonosis with a large animal reservoir, it can be spread to humans through contaminated food or water, animal contact (most commonly with pets or farm animals), or contaminated environments.Typhoid Salmonella and nontyphoid Salmonella (NTS) are two categories into which *Salmonella* strains can be divided based on the clinical characteristics of human salmonellosis [23].Enteric fever, gastroenteritis, bacteremia and associated extraintestinal consequences, and chronic carrier state are the four distinct clinical manifestations of human infections [28].

2.4.5. *Vibrio spp*

Rod-shaped, gram-negative bacteria called *Vibrio spp* are found naturally in freshwater, estuaries, and marine habitats. Cholera, a severe diarrheal illness that is usually spread by contaminated water and person-to-person contact, can be brought on by *Vibrio cholerae* [23] . If left untreated, cholera can be lethal very fast. Not only cholera *Vibrio spp*. are the bacteria that cause vibriosis, an infection typically contracted by exposure to sea water or by eating raw or undercooked infected seafood. Examples of these bacteria are *Vibrio parahaemolyticus*, *Vibrio alginolyticus, and Vibrio vulnificus* [24].

2.4.6 *Yersinia spp*

The bacterial family Yersiniaceae includes the genus Yersinia. According to Ryan and Ray (2004), the Yersinia species are facultative anaerobes, which are Gram-negative coccobacilli bacteria with a diameter of fractions of a micrometer and a length of a few micrometers. Human lymphoid tissue is a suitable place for several pathogenic Yersinia species, such as *Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica*, which can all lead to invasive, systemic illness [27]. Animal invasiveness varies, but in general, Y. pestis>Y. pseudotuberculosis>Y. enterocolitica is the order in which they are most invasive. A unique Y. enterocolitica serotype (0:8) is as virulent as or more so than Y. pseudotuberculosis in mice [25].

2.5. Public Health Implications of Antibiotic Resistance from Aquaculture

The public health consequences of antibiotic resistance stemming from aquaculture are complex , extensive, and multifaceted. These results encompass direct health dangers due to exposure to resistant bacteria, indirect impacts via contamination of food and the environment.

2.5.1.Spread of Resistant Bacteria to Humans

One of the most straightforward avenues through which antibiotic resistance in aquaculture impacts human health is by the transmission of resistant bacteria or resistance genes from fish and aquatic environments to humans. Individuals can be exposed in various ways.

2.5.2.Eating contaminated fish

If fish are insufficiently cooked, resistant bacteria can survive and enter the human digestive system. These bacteria may persist as part of the gut microbiome or transfer their resistance genes to other bacteria in the bod including potentially harmful pathogens [7].

2.5.3. Handling fish and pond water

Farmers, processors, and market workers often come in contact with contaminated fish, sediments, or pond water. Without maintaining proper hygiene and protective measures, resistant bacteria has the ability to colonize human skin or enter through minor wounds [10].

2.5.4.Environmental exposure

Pond effluents and water runoff sometimes coupled with household water supplies in many aquaculture-dependent nations, like Bangladesh. Unknowingly, people who use these waters for irrigation, cleaning, and bathing may come into contact with resistant microorganisms [10].These frequent exposures increase the likelihood that antibiotic-resistant bacteria will proliferate in human populations over time, making common infections more challenging to treat [11].

2.5.5.Transfer of Resistance genes to human

Horizontal gene transfer (HGT), a natural method by which bacteria exchange genetic information can result in the transmission of resistance genes from aquatic bacteria to human diseases [11].Genes that transfer resistance from benign ambient bacteria to pathogenic species such as *Salmonella, Vibrio cholerae*, or E. *coli* can cause illnesses that are resistant to traditional therapies.Similar resistance genes, including *tetA, sul1, and blaTEM* have been identified in both human clinical samples and bacteria from aquaculture ponds [14].This overlapping suggests that aquaculture environments could act as breeding grounds and reservoirs for resistant traits that subsequently manifest in communities and hospitals.

2.5.6.Food Safety and Antibiotic Residues

The finding of antibiotic residues in fish tissues is a significant problem for food safety. Fish may accumulate trace amounts of antibiotics due to overuse or failure to adhere to the prescribed withdrawal periods [12].Consuming these residues may cause a number of issues.Allergic reactions: Sensitive people may experience allergic reactions to antibiotic residues,particularly those originating from sulfonamide or β-lactam medications.Gut microbiota disruption: The natural balance of good bacteria in the human digestive tract can be upset by prolonged exposure to low concentrations of antibiotics through food, which may result in digestive problems or weakened immunity [14,15].Research has frequently found fluoroquinolone, chloramphenicol, and oxytetracycline residues in aquaculture fish in Bangladesh and other developing nations, sometimes exceeding globally accepted limits [20].This emphasizes how important it is to have more oversight and regulation.

2.5.7.Occupational Health Risks

Farmers, fish handlers, feed manufacturers and workers in processing facilities are among those directly involved in aquaculture who run the danger of coming into contact with

antibiotic-resistant bacteria.According to research, farm workers frequently have resistant bacterial strains that are similar to those in the ponds they manage [18].They may breath these bacteria when performing chores like feeding, harvesting, or cleaning the ponds, or they may enter their bodies through minor incisions.Asymptomatic colonization, in which resistant bacteria live harmlessly in workers' bodies but later cause diseases or spread to family members and the larger population, can occur with continued exposure [17,18].This type of silent transmission poses a serious threat to public health, especially in areas with limited access to medical care.

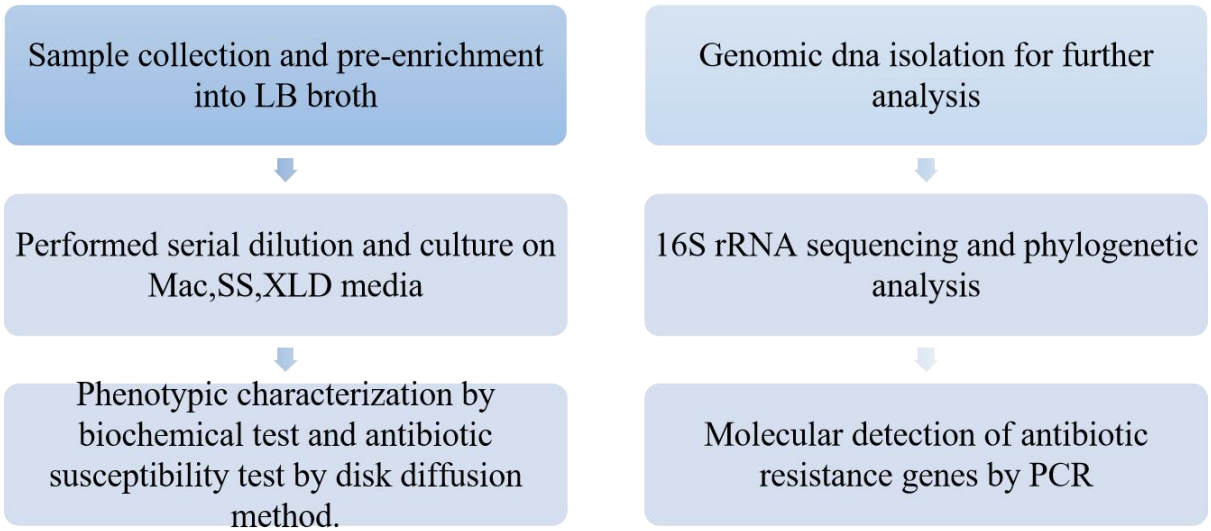
2.6. Strategies for Mitigation and Sustainable Practices to Lower Antibiotic Resistance in Aquaculture

In order to combat antibiotic resistance in aquaculture, treatment-based farming must give way to a more ethical and long-term approach for health management. Using veterinary guidance, avoiding growth-promoting or preventative treatments, following recommended dosages and withdrawal times are all important ways to reduce the needless use of antibiotics [24].Simultaneously, enforcing national standards, keeping an eye out for residues and putting surveillance procedures in place guarantee the safe and responsible use of antibiotics.Better water quality, controlling stocking density, improving hygiene and implementing stringent biosecurity measures are examples of sustainable agricultural methods that greatly reduce disease outbreaks and the initial need for antibiotics [21,22].Vaccines, probiotics, immunostimulants, and herbal therapies are examples of environmentally acceptable ways to increase fish immunity and control bacterial infections without causing resistance.By naturally reducing trash and disease levels, integrated agricultural techniques like multitrophic aquaculture also improve ecosystems. Since many small-scale producers lack sufficient training in the proper use of antibiotics and disease management, teaching farmers is also essential [17].Aquaculture may advance toward a safer and more sustainable framework that protects not only fish health but also environmental integrity and public health by combining responsible medicine use, creative disease-prevention techniques, strict laws, and community education [28].

Chapter-3: Materials and Methods

3.Materials and Methods

Methodology of the research



3.1. Study area and sample collection

A total of 24 Thai Pangas were collected from different local areas of Noakhali zone under the division of Chittagong in Bangladesh at different time interval and packed in sterile polyethylene bags. These places were:

Farm	Number of Sample (Thai Pangas)	Collection Area	Date of Collection
Integrated 1	3	Dharmapur,Hajirhat,Noakhali Sadar	14/05/25
Integrated 2	3	Gajir khamar,Thanarhat,Noakhali Sadar	01/06/25

Integrated 3	3	Bismillah Farm, Sonapur,Noakhali Sadar	18/06/25
Integrated 4	3	Akram member bari,Kalitara Bazar road,Noakhali Sadar	06/07/25
Non Integrated 1	3	Italy Market, Kabirhat,Noakhali	28/07/25
Non Integrated 2	3	Bahar Miyar plant, Subarna Agro,Sonapur,Noakhali Sadar	17/08/25
Non Integrated 3	3	Cheuakhali, Subarnachar,Noakhali	11/09/25
Non Integrated 4	3	Shibpur,Badherhat,Noakhali Sadar	03/10/25

Figure 3.1: Sample Collection from Different Farms

This study was carried out in the laboratory of the Department of Microbiology at Noakhali Science and Technology University (NSTU). Gut samples of Thai Pangas (Pangasius hypophthalmus) were collected from small- and medium-scale integrated fish–poultry farms

located in different areas of Noakhali.During sample collection, sterile instruments were used, and strict aseptic techniques had been followed to prevent contamination and ensure the reliability of microbiological analyses.

3.2. Sample transport and processing

Thai Pangas (Pangasius hypophthalmus) samples were collected from various integrated poultry–fish farming sites across the Noakhali region. Immediately after collection, the fish was placed in sterile, insulated containers and transported on ice to the Laboratory at the Department of Microbiology ensuring that cold chain conditions are maintained to preserve sample integrity.The fish samples were surface sterilized with 70% ethanol upon arrival at the lab, and the intestinal contents were extracted through aseptic dissection.Before being isolated and subjected to additional examination, the obtained gut samples were placed in sterile nutrition broth or enrichment media known as Lureia Burtani Broth (LB) and cultured for 24 hours at 37°C to encourage bacterial growth.In this instance, 50 ml of LB media was enriched with 5g of each fish's gut sample.

Figure 3.2: Sample Processing and Enrichment

3.2.1 Dilution of samples: Serial dilutions involved progressively diluting a sample in a sterile solution like saline water.This helped to obtain a manageable number of bacteria for

isolating individual colonies on agar plates. Typically, a tenfold dilution series was prepared. This means for every 1 mL of the sample, 9 mL of sterile diluent was added. This process was repeated for several tubes to achieve desired dilutions.A specific amount (usually 0.1 mL) from each dilution was then spread onto appropriate agar media to allow for bacterial growth.

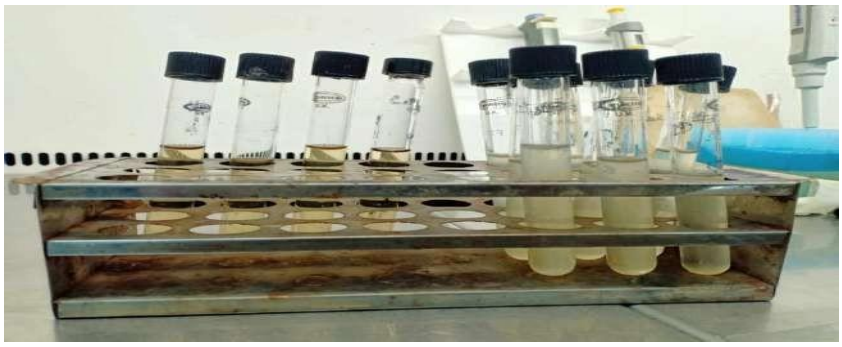


Figure 3.3: Sample dilution process

3.3. Isolation of potential pathogenic bacteria

Each dilution was spread on separate plates of MacConkey Agar (Oxoid, UK),Shigella Salmonella (SS) agar,XLD agar and was incubated at 37°C for 24 hours. After 24 hours, presumptive bacterial colonies on respective plates were found having distinct colony morphologies including pink.mucoid, purple, yellow,black centered etc. Some colonies that are closely related to our desired bacteria were selected and picked to subculture onto MacConkey agar, SS agar and XLD agar.

Figure 3.4: Streaking Technique for the purpose of Isolation of Bacteria

3.4. Stock Preparation

After Subculturing the desired colonies into media plates to get pure culture, stocking is necessary for further analysis of microbial colonies, usually organisms.

Procedure

3.4 1. Labeling: A marker pen was used to clearly label the sterile tubes or cryovials with the organism ID and date.

3.4.2. Preparation of the cryovials/tubes with broth: Using sterile technique 800 microliters (µL) of TSB broth was transferred into each sterile tube or cryovial using a micropipette with a sterile tip.

3.4.3. Addition of Glycerol: Carefully 300 microliters (µL) of glycerol was added to each tube or cryovial using a new sterile pipette tip. Glycerol acts as a cryoprotectant, helping to protect the organisms during freezing.

3.4.3. Sterilization of the cotton swab: Holding the cotton swab with forceps and ignited the end using a Bunsen burner or another sterilization method.The flame was burned for a few seconds, then carefully waft it in the air to cool down slightly.

3.4.4. Transferring Organisms:The source of subculture plate hold briefly near the flame and gently touched the surface of the growing organisms with the cooled sterile cotton swab.

3.4.5. Inoculation of the culture: The cap from the labeled tube or cryovial was removed and inoculated it with the cotton swab by gently rubbing the swab against the inside of the container. It was made to deposit a representative sample of the organisms into the broth.

3. 4. 6. Mixing: Recapped the tube or cryovial securely and gently mixed the contents by inverting the vial a few times. Bubbles formation was technically avoided.

3.4.7. Freezing (for long-term storage): For long-term storage,the tubes or cryovials were stored in a cryogenic freezer at -80°C or colder.

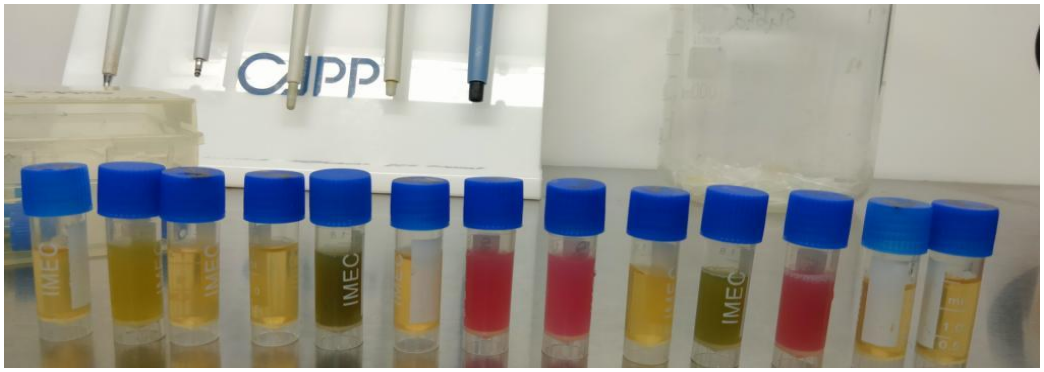
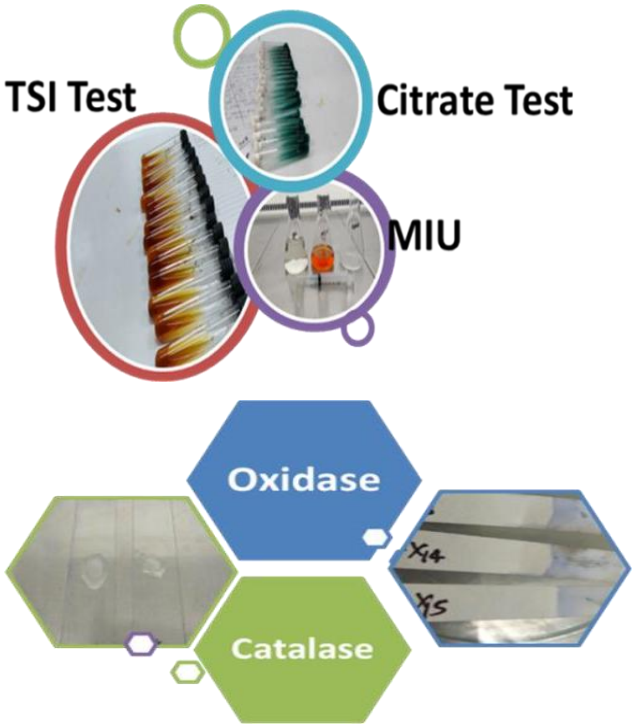


Figure 3.5: Stock culture for further analysis

3.5 Biochemical Test (Identification of pathogenic bacteria) :The identification of colonies of the isolates were done by performing various biochemical tests. For Gram (+) ve bacteria and Gram (-) ve bacteria, Citrate utilization test, MIU (motality, indole and urease) test, oxidase test, Triple sugar iron(TSI) (acid slant/acid butt/gas) test were performed. All the tests were performed according to the standard protocol as described in Bergey's Manual (Brenner et al., n.d.).



3.5.1 Citrate utilization test

100ml of distilled water was used to dissolve roughly 2.4g of citrate agar. Each tube was filled with about 3 ml of citrate medium, covered, sterilized, and allowed to cool in an

inclined position. The organisms were streaked once across the surface of the tubes to inoculate them. The usage of the citrate is indicated by the change from green to blue.

3.5.2 MIU(motility, indole,urease)test

MIU semi solid agar is used for identification of bacteria with motility test, urease and indole production.

Motility: Isolates were stabbed into the middle of the tubes and incubate at 37°C for 24 hours.Motility positive is indicated by a diffuse from the stabbing point.

Urease: In this test urea was mixed with MIU media through 0.45nm filter paper with a sterile syringe. The presence of urease enzyme can be detected containing the PH indicator phenol red, as the substrate urea is spilt into its products, phenol red turn to deep pink. This is positive reaction for the presence of urease.

Indole: Indole production was detected by Kovac's reagent which contained 4(p)-dimethyl amino benzaldehyde (1 g), isoamyl alcohol (15ml) and hydrochloric acid. Kovac's reagent dropped through a dropper (three drop) of the motility media containing tube and indole production was indicated by a reddening in the top of the tube.

3.5.3. Oxidase Test

The oxidase test was used to find out if the bacteria had the cytochrome oxidase enzyme. The Gaby and Hadley oxidase test reagent was applied to a little piece of filter paper, which was then allowed to dry. A well-isolated colony from a pure 24hour culture was selected, rubbed onto filter paper using an inoculating loop, and its color was monitored (Shields and Cathcart, 2010).

3.5.4.Catalase Test

The catalase test was used to find out if the bacteria could break down hydrogen peroxide by generating the catalase enzyme. A petri dish was filled with a tiny slide. A tiny quantity of bacteria from a 24-hour pure culture was added to the microscopic slide using a sterile inoculating loop. Using a dropper, one drop of 3% H_2O_2 was added to the organism on the microscopic slide, and the organism was watched to see if any bubbles formed right away. Sherman and Cappuccino, 2005).

3.5.5.Triple Sugar Iron(TSI) test

A microbiological test called the Triple Sugar Iron, or TSI, is used to evaluate an organism's capacity to ferment sugars and generate hydrogen sulfide. Enteric bacteria are selectively identified using it. First, the top of a well-isolated colony with a sterile inoculation needle was touched Next,by stabbing and streaking, inoculation was used to introduce the test organism into the media. After that, the tube was incubated for 24 hours at 37°C. The fermentation of lactose (or sucrose) results in a strong acid production, which is what causes the phenol red indicator in butt and slant to turn yellow. Gases released by certain organisms cause bubbles in the medium. If lactose is not fermented but some glucose is, the oxygen-deficient butt will appear yellow; on the other hand, if the organism oxidizes the acid to produce carbon dioxide and water, the slant will turn red. When H2S is produced, ferrous sulfide's black hue becomes noticeable.

3.6. Procedure of Antibiogram

3.6.1. Preparation of Mueller-Hinton plate

Muller-Hinton agar (MH agar) plate for each organism to be tested was allowed to come to room temperature.It was advisable to keep the plates in the plastic sleeve while they acclimated to prevent condensation. If any liquid was visible on the agar's surface, the plate was inverted, placed on its lid with a jar, to let the excess liquid drain and evaporate from the agar.The plates were then placed either in a 35°C incubator or in a laminar flow hood at room temperature until they dried, which typically took 10 to 30 minutes. Each MH agar plate was properly labeled corresponding to the organism being tested.

3.6.2. Preparation of Inoculum:

Using a sterile inoculating loop or needle, four or five isolated colonies of the organism to be tested were transferred into 10 mL of sterile saline, and the tube was vortexed to create a smooth, uniform suspension. The turbidity of the suspension was then adjusted to match a 0.5 McFarland standard by adding more organism if it appeared too light or diluting with sterile saline if it was too heavy. The prepared suspension was used within 15 minutes of preparation.

3.6.3 Inoculation of MH plate:

A sterile swab was immersed in the inoculum tube. The swab was then rotated against the interior wall of the tube, above the liquid level, with firm pressure applied to eliminate any surplus liquid, ensuring it was not excessively wet. The surface of a dried Mueller-Hinton (MH) agar plate was inoculated by streaking the swab three times across the entire agar surface, turning the plate about 60 degrees each time to ensure an even distribution of the inoculum. Lastly, the edge of the plate was swabbed to capture any remaining excess liquid.

3.6.4. Placing antibiotic disks on the agar

Antibiotic-impregnated discs were applied to the surface of the inoculated plate with sterile forceps. In each plate three to five discs were placed. All discs were gently pressed down onto the agar with forceps to ensure complete contact with the agar surface. Within 15 minutes after the discs were applied, the plates were inverted and placed in an incubator at 37°C.

3.6.5. Interpretation of agar plates

After 18-20 hours of incubation, each plate was examined for the zone of inhibition, uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition (judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter. The sizes of zones of inhibition were interpreted according to the performance standards for antimicrobial susceptibility testing and an organism were reported as susceptible, intermediate or resistant to various antibiotics based on the CLSI,2021.

3.7. Preparation of Template DNA:

1.01 ml portion of each bacterial culture in TSB broth was transferred to the labeled microcentrifuge tube.

2. Then centrifuged the tube for 5 min at 14,000 rpm(10,000×g).

3.Discarded the supernatant and washed the pellet by adding 1 ml of PBS solution.

4.Mixed properly then further centrifuged the tube for 5 min at 14000 rpm (10,000×g).

5.After final washing removed the supernatant and resuspended the pellet with 350 µL of nuclease free water.

6.Then the suspension boiled for 5 minutes at 100°C using heat block to lyse the bacteria followed by quick cooling on ice for 3 min.

7. After that, centrifugation was done at 10,000 g for 10 min.

8.The supernatant was collected carefully and transferred to a new sterile tube with appropriate label then stored at -20°C until used as a template for PCR reaction.

3.8. PCR Amplification of the 16S rRNA Gene

3.8.1 The nearly complete 16S rRNA gene which measures around 1.5 kilobases in size was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′).These primers are commonly utilized for bacterial identification as they focus on conserved regions surrounding variable domains within the 16S rRNA gene,enabling broad amplification across various bacterial taxa.

3.8.2. PCR amplification was conducted in a total reaction volume of 25 µL which consisted of genomic DNA as the template, forward and reverse primers, deoxynucleotide triphosphates (dNTPs), $MgCl_2$, Taq DNA polymerase, and the designated reaction buffer. All components were prepared with sterile, nuclease-free water to avoid contamination.

3.8.3.The thermal cycling parameters were optimized as follows: an initial denaturation phase at 95°C for 3 minutes to ensure the complete separation of the DNA strands, followed by 30 amplification cycles that included denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds.To enable the complete synthesis of any residual incomplete DNA strands, a last extension at 72°C for 10 minutes was included.

3.8.4. Using electrophoresis on a 1% agarose gel made in 1× TAE buffer and stained with ethidium bromide, the amplified PCR products were evaluated.To verify the length of the amplified fragment, a 1 kb DNA ladder was used as a molecular size marker.The presence of a distinct band of around 1.5 kb which matches the expected size of the 16S rRNA gene was confirmed by seeing the gels under ultraviolet (UV) light using a gel documentation system.

3.9. Purification and Sequencing of PCR Products

- Following amplification any leftover primers, nucleotides, enzymes, and buffer components that would interfere with the sequencing reaction were removed from the PCR products.
- The same primers (27F and 1492R) used for the amplification process were used to sequence the purified 16S rRNA gene segments in both orientations.An automated DNA sequencer like the ABI 3730XL Genetic Analyzer was used to perform the sequencing procedures using the Sanger dideoxy technique.The quality and reliability of the final consensus sequence were improved by this dual-direction approach, which made it easier to obtain overlapping sequence reads.
- The raw sequence chromatograms were edited to remove low-quality ends and remaining primer sequences after being visually inspected to verify the quality of base-calling. BioEdit, ChromasPro, or MEGA tools were then used to combine the forward and reverse reads into a continuous consensus sequence. To ensure accuracy, any unclear base calls were manually adjusted by comparing the electropherogram peaks from both directions. For further investigations, the high-quality consensus sequence that was produced was used.

3.10. Sequence Analysis and Phylogenetic Tree Construction

- The closest phylogenetic relatives and the taxonomic identity of the bacterial isolates were determined by bioinformatic analyses of the high-quality consensus sequences of the 16S rRNA gene acquired by Sanger sequencing.
- First, the BLASTn method was used to compare each sequence with the National Center for Biotechnology Information's (NCBI) nucleotide database.By identifying the sequences with the highest degree of similarity, this stage allowed for a preliminary determination of the isolate's potential genus and species. Sequences were classified as belonging to the same genus if they had ≥97% similarity to known reference strains, and as possible members of the same species if they had ≥99% similarity.

- Multiple sequence alignments were carried out using either the ClustalW or MUSCLE algorithms from the MEGA X software program in order to validate these identifications and investigate the evolutionary relationships between the isolates and their related species. To guarantee thorough phylogenetic coverage, representative 16S rRNA sequences of closely related species and type strains were obtained from the GenBank database and incorporated into the alignment.

★ The confirmed 16S rRNA gene sequences were uploaded to the National Center for Biotechnology Information (NCBI) GenBank database in order to guarantee that the molecular data generated in this study will be accessible for future reference and comparative investigations. Every sequence was carefully reviewed to guarantee its quality, accuracy, and completeness prior to submission. In compliance with GenBank submission rules, information about each isolate was collected, including sample origin, geographic location, collection date, and organism details.

★ Sequence data were uploaded through the NCBI's BankIt submission system after being prepared in FASTA format.To enhance the readability and traceability of the sequence records, further annotations such gene name, isolate identification, and methodological details were included throughout the submission process.Each sequence was assigned a distinct GenBank accession number, which serves as a permanent and publicly accessible identification, after passing the NCBI's quality checks.

★ In order to allow other researchers to verify the results, compare related sequences, or utilize the given data in more extensive phylogenetic or taxonomic study, these accession numbers were incorporated into the thesis's results and discussion sections. The study contributes to the growing body of molecular data available for microbial identification and evolutionary research by storing the sequences in an accredited international repository.

3.11. Molecular methods for detection of Antibiotic Resistance Genes

3.11.1. Primer Designs used for the study

Antibiotic Resistance Gene	Sequence (5'-3'	Annealing Temperature (°C)	Amplicon Size (bp)	References	
tetA	F:GGTTCACTCGAACGACGTCA R:CTGTCCGACAAGTTGCATGA	53.07	950	[36]	
blaCTX-M	F:ATGTGCAGYACCAGTAARGTKATGGC R:TGGGTRAARTARGTSACCAGAAYCAGCGG	59	500		
blaTEM	F:CGCCGCATACACTATTCTCAGAATGA R:ACGCTCACCGGCTCCAGATTTAT	57.05	900		
sul1	F:GGCCGATGAGATCAGACGTA R:TTTGAAGGTTCGACAGCACG	50.06	800	[39]	
qnr	F:ACGCCAGGATTTGAGCGACAGC R:CGCTGAGGTTGGCATTGCTCCA	58	457		

3.11.2. Preparation of PCR mixture

To check whether a bacterial sample carries an antibiotic-resistance gene, the DNA was isolated by boil dna lysis procedure .It was combined with the ingredients needed for PCR such as Taq Polymerase,MgCl2, nuclease free water and primer.These components are mixed by spinning so the reaction can proceed efficiently in the thermocycler.

3.11.3. Amplification in the thermocycler

PCR worked by repeatedly heating and cooling the reaction in controlled cycles.Initial heating:The reaction was warmed to a high temperature to separate DNA strands and prepare the polymerase for copying.Denaturation:Each cycle began with another brief high-temperature step that pulled the two DNA strands apart.Annealing:The temperature was then lowered so the primers could attach to their matching sequences on the single-stranded

DNA.Extension:The temperature shifts to the optimal range for the polymerase to build new DNA strands, starting from each primer.Final extension and hold: After the cycling was complete, the reaction was given a final warm period to finish any remaining DNA synthesis, and then kept cool until analysis.

3.11.4 Detection of the Amplified genes

To see whether the target gene was successfully amplified, the PCR products are separated by size using gel electrophoresis. DNA fragments move through a gel when an electric current is applied, and a dye allows the separated fragments to be visualized. If a band appears at the characteristic size expected for the resistance gene, it suggests that the gene was present in the original DNA sample.

Chapter-4: Results

4.Results

Table 4.1 : Colony Morphology and Characteristics of all isolates detected from samples

Farm No	Sample No	Isolate ID	Culture Media	Colony morphology	Presumptive Organism
1	1	F1S1A	Mac	Pink,Convex	*E coli*
		F1S1B	Mac	Large reddish mucoid	*Klebsiella pneumoniae*
		F1S1C	SS agar	Colorless black centered	*Proteus mirabillis*
		F1S1D	Mac	Transparent, Colorless	*W chitiniclastica*
		F1S1E	XLD	Large black centered	*Salmonella spp*
	2	F1S2A	Mac	Pink,Convex	*E coli*
		F1S2B	Mac	Pale,Colorless	*W chitiniclsstica*
		F1S2C	Mac	Large reddish mucoid	*Klebsiella spp*
		F1S2D	SS agar	Black centered colorless	*Proteus mirabillis*
		F1S2E	SS agar	Large black centered	*Salmonella enterica*
	3	F1S3A	Mac	Large, Mucoid	*Klebsiella pneumoniae*

		F1S3B	Mac	Pink,Convex	*E coli*
		F1S3C	SS	Black centered	*Proteus mirabillis*
		F1S3D	XLD	Blackish	*Salmonella enterica*
2	1	F2S1A	Mac	Reddish mucoid	*Klebsiella pneumoniae*
		F2S1B	Mac	Pale,Convex	*Aeromonas hydrophila*
		F2S1C	XLD	Black centered	*Proteus mirabillis*
		F2S1D	Mac	Reddish pink	*E coli*
		F2S1E	XLD	Large black centered	*Salmonella sp*
	2	F2S2A	Mac	Red mucoid	*Klebsiella pneumoniae*
		F2S2B	SS agar	Blackish	*Salmonella enterica*
		F2S2C	SS agar	Transparent, Colorless	*Shigella dysenteriae*
		F2S2D	XLD	Black centered	*Proteus mirabillis*
		F2S2E	Mac	Reddish pink	*E coli*
	3	F2S3A	Mac	Mucoid, Pink	*Klebsiella pneumoniae*
		F2S3B	Mac	Pink,Convex	*E coli*

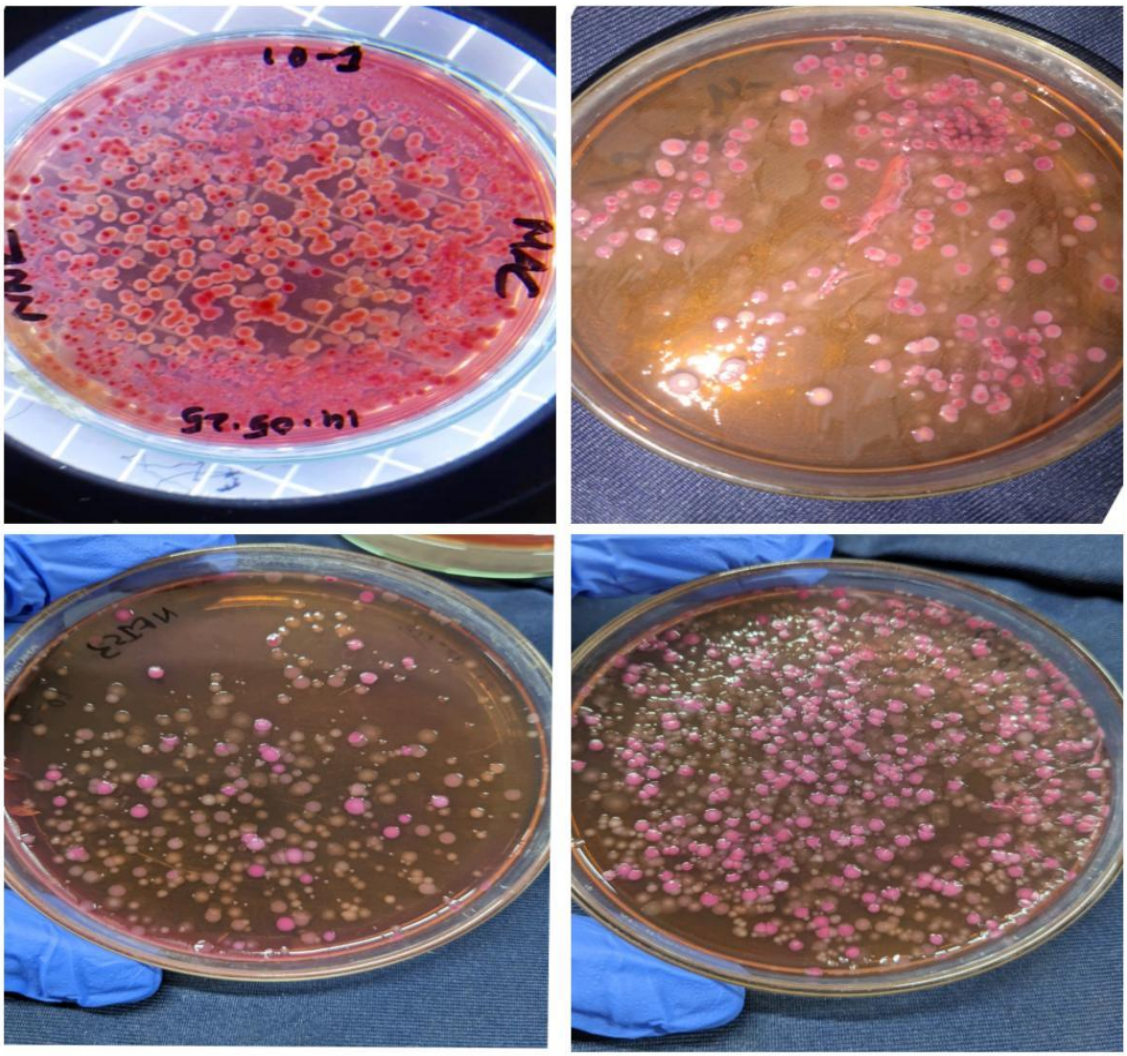
		F2S3C	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		F2S3D	XLD	Blackish	*Salmonella enterica*
3	1	F3S1A	Mac	Mucoid,Pink	*Klebsiella pneumoniae*
		F3S1B	Mac	Pale,Convex	*W chitinicolistica*
		F3S1C	XLD	Black centered	*Proteus mirabillis*
		F3S1D	Mac	Pink,Convex	*E coli*
		F3S1E	Mac	Pale,Opaque	*Aeromonas hydrophila*
	2	F3S2A	Mac	Pink,Convex	*E coli*
		F3S2B	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		F3S2C	Mac	Pink,Convex	*E coli*
		F3S2D	XLD	Black centerd colorless	*Proteus mirabillis*
		F3S2E	SS agar	Transparent, Colorless	*Shigella dysenteriae*
	3	F3S3A	Mac	Pink,Convex	*E coli*
		F3S3B	XLD	Blackish	*Salmonella enterica*
		F3S3C	SS	Black centered	*Proteus mirabillis*

4	1	F4S1A	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		F4S1B	Mac	Pale,Opaque	*Aeromonas hydrophila*
		F4S1C	Mac	Pale,Convex	*W chitinicolistica*
		F4S1D	Mac	Round, Colorless	*Aeromonas hydrophila*
		F4S1E	SS agar	Reddish pink	*E coli*
	2	F4S2A	SS agar	Yellow,Convex	*E coli*
		F4S2B	SS agar	Black centered	*Proteus mirabillis*
		F4S2C	Mac	Transparent	*Enterbacter cloacae*
		F4S2D	Mac	Pale,Convex	*Aeromonas hydrophila*
		F4S2E	XLD	Large black centered	*Salmonella enterica*
	3	F4S3A	Mac	Pink,Convex	E coli
		F4S3B	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		F4S3C	SS	Black centered	*Salmonella enterica*
		F4S3D	SS	Black centered	*Proteus mirabillis*
	1	NF1S1A	Mac	Pink,Convex	*E coli*

1		NF1S1B	Mac	Pink mucoid	*Klebsiella spp*
		NF1S1C	Mac	Pale,opaque	*Aeromonas spp*
		NF1S1D	SS agar	Black centered	*Proteus mirabillis*
	2	NF1S2A	XLD	Large black centered	*Salmonella enterica*
		NF1S2B	XLD	Black centered	*Proteus mirabillis*
		NF1S2C	Mac	Pink,Convex	*E coli*
	3	NF1S3A	Mac	Pink mucoid	*Klebsiella pneumoniae*
		NF1S3B	Mac	Pink,Convex	*E coli*
		NF1S3C		Pale,Convex	*Proteus mirabillis*
2	1	NF2S1A	Mac	Pink,Convex	*E coli*
		NF2S1B	SS agar	Transparent, Colorless	*Shigella spp*
		NF2S1C	SS agar	Black centered	*Proteus mirabillis*
	2	NF2S2A	Mac	Round,Colorless	*Aeromonas hydrophila*
		NF2S2B	Mac	Pink,Convex	*E coli*
		NF2S2C	Mac	Pink mucoid	*Klebsiella pneumoniae*

		NF2S2D	Mac	Transparent	*Enterobacter spp*
	3	NF2S3A	Mac	Pink,Convex	*E coli*
		NF2S3B	XLD	Black centerd	*Proteus mirabillis*
		NF2S3C	Mac	Pink,Convex	*E coli*
3	1	NF3S1A	XLD	Large black colony	*Proteus mirabillis*
		NF3S1B	Mac	Pink,Convex	*E coli*
	2	NF3S2A	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		NF3S2B	Mac	Pale,Opaque	*Aeromonas hydrophila*
	3	NF3S3A	Mac	Pink,Convex	*E coli*
		NF3S3B	SS agar	Slightly pink	*Shigella dysenteriae*
		NF3S3C	SS agar	Pink,Mucoid	*E coli*
4	1	NF4S1A	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		NF4S1B	SS agar	Black centered	*Proteus mirabillis*
		NF4S1C	Mac	Pink,Convex	*E coli*
	2	NF4S2A	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		NF4S2B	SS agar	Black centered	*Proteus*

					mirabillis
	3	NF4S3A	Mac	Pink, Convex	*E coli*
		NF4S3B	Mac	Pink,Mucoid	*Klebsiella pneumoniae*
		NF4S3C	XLD	Black centered	*Proteus mirabillis*
		NF4S3D	Mac	Pale,Opaque	*Aeromonas hydrophila*



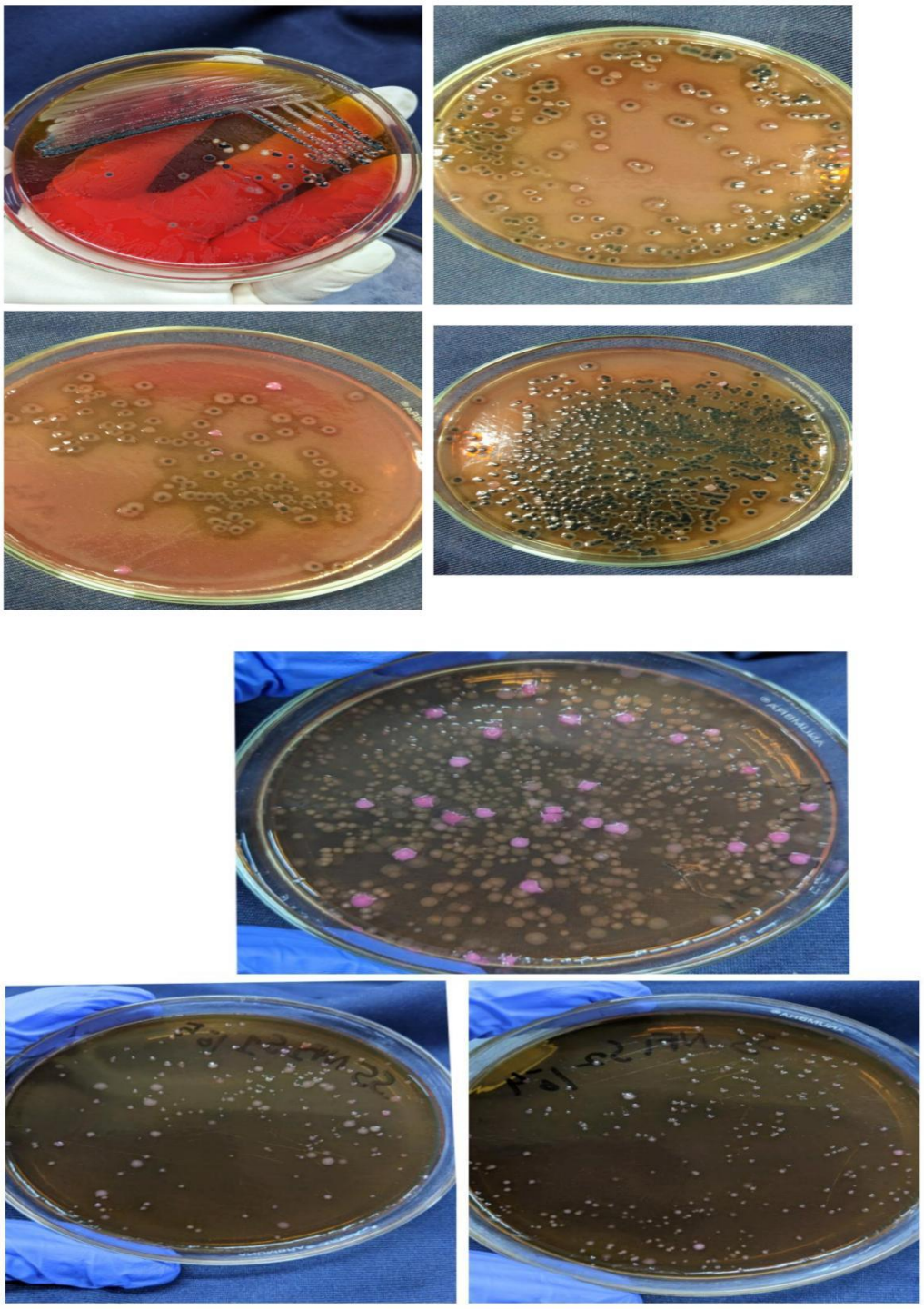
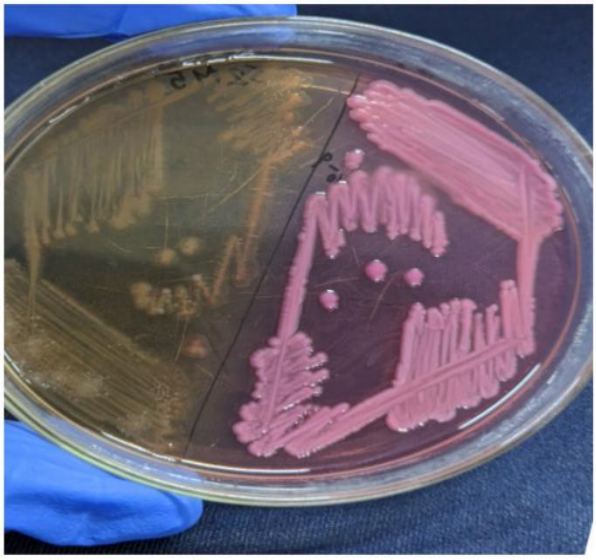
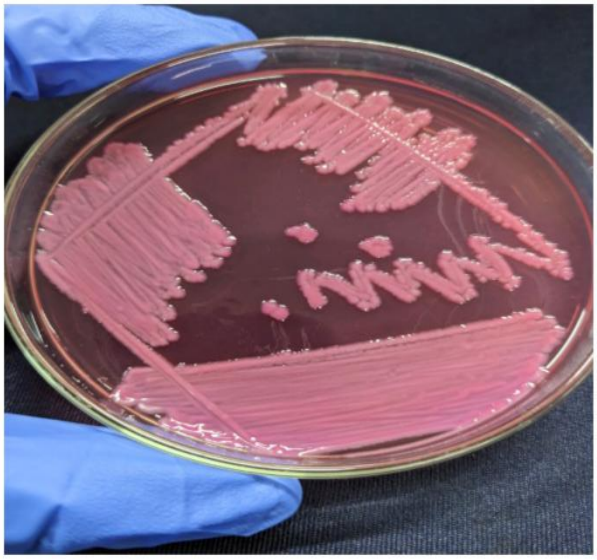
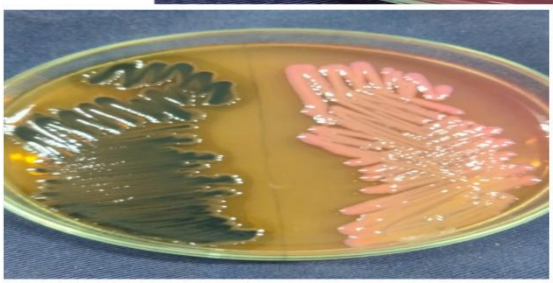


Figure 4.1: Mixed culture in Mac XLD and SS agar







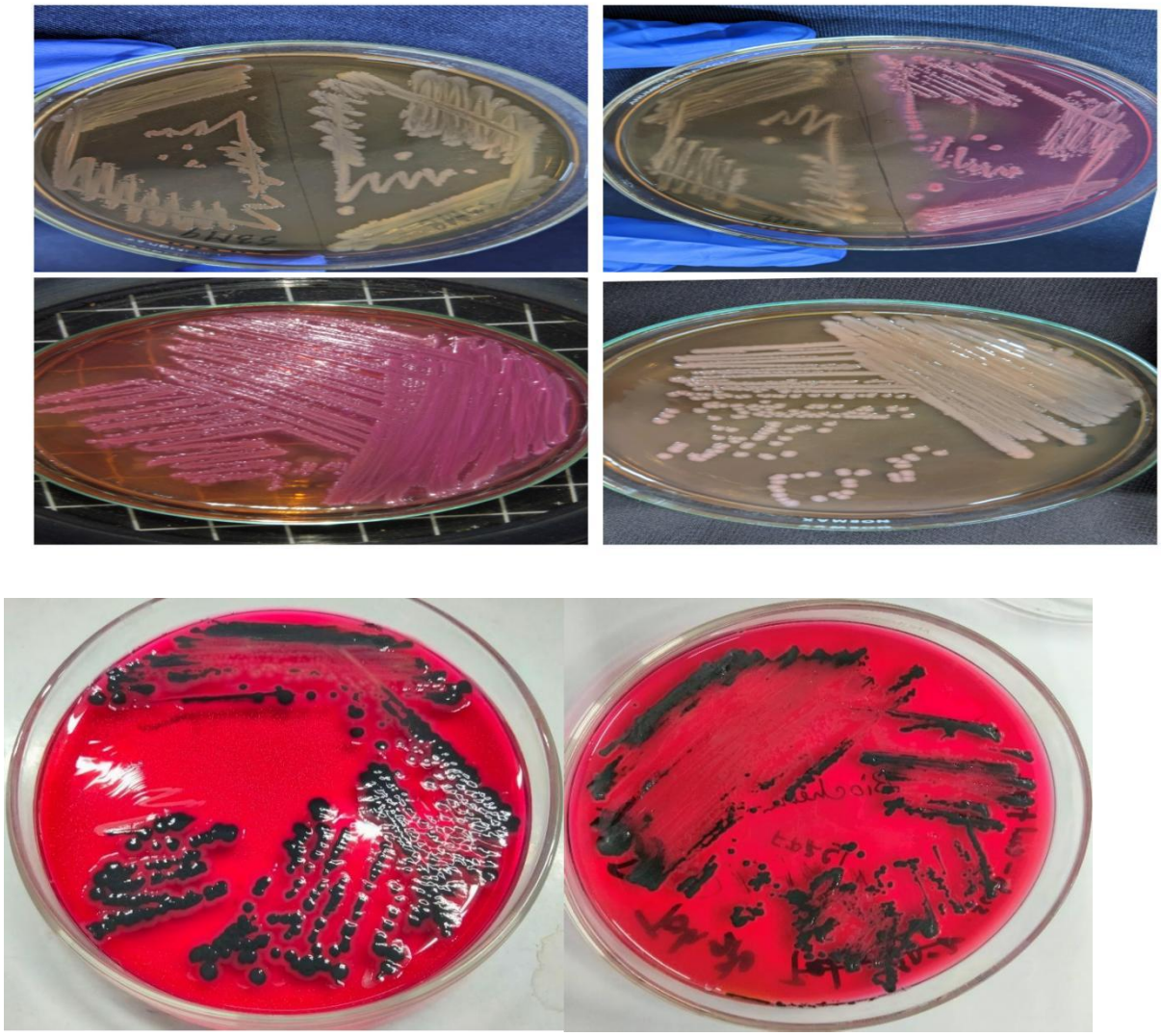


Figure 4.2: Pure Culture in Mac,SS,XLD agar

Table 4.2: Biochemical Test results of all isolates

Sample ID	MIU			TSI				Citrate	Oxidase	Catalase	Presumptive Organism
	M	I	U	Slant	Butt	H2S	Gas				
F1S1A	+	+	-	A	A	-	+	-	-	+	***Escherichia coli***
F1S1B	-	-	+	A	A	-	+	+	-	+	***Klebsiella pneumoniae***

F1S1C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F1S1D	-	+	-	K	A	-	-	-	-	-	***Wohlfahrtiimonas chitiniclastica***
F1S1E	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***

F1S2A	+	+	-	A	A	-	+	-	-	+	***Escherichia coli***
F1S2B	+	+	+	A	A	-	+	+	+	+	***A hydrophila***
F1S2C	-	+	+	A	A	-	+	+	-	+	***Klebsiella spp***
F1S2D	+	+	+	K	A	+	-	-	-	+	***Proteus vulgaris***
F1S1E	+	-	-	A	A	+	-	-	-	+	***Salmonella***
F2S1A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F2S1B	+	-	-	A	A	+	+	-	-	+	***Salmonella enterica***
F2S1C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F2S1D	+	+	-	A	A	-	+	-	-	+	***E coli***
F2S1E	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
F2S2A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F2S2B	+	+	-	A	A	+	-	-	-	+	***Salmonella***

											enterica
F2S2C	+	+	-	A	A	-	+	-	-	+	***E coli***
F2S2D	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F2S2E	+	+	-	A	A	-	+	-	-	+	***E coli***
F2S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
F2S3B	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
F2S3C	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F2S3D	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***

F3S1A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F3S1B	+	-	+	A	A	+	-	+	-	+	***Aeromonas hydrophila***
F3S1C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F3S1D	+	+	-	A	A	-	+	-	-	+	***Escherichia coli***
F3S1E	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***

F3S2A	+	+	-	A	A	-	+	-	-	+	***Escherichia coli***

F3S2B	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F3S2C	-	-	+	K	K	-	-	+	-	+	***Acinetobactor sp.***
F3S2D	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F3S2E	-	+	-	K	A	-	-	-	-	-	***Wohlfahrtiimonas chitiniclastica***
F3S3A	+	+	-	A	A	-	+	-	-	+	**E coli**
F3S3B	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F3S3C	+	+	-	A	A	-	+	-	-	+	***Ecoli***

F4S1A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
F4S1B	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***
F4S1C	+	-	+	A	A	+	-	+	-	+	***W chitiniclastica***
F4S1D	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
F4S1E	+	-	-	A	A	-	+	+	-	-	***E coli***

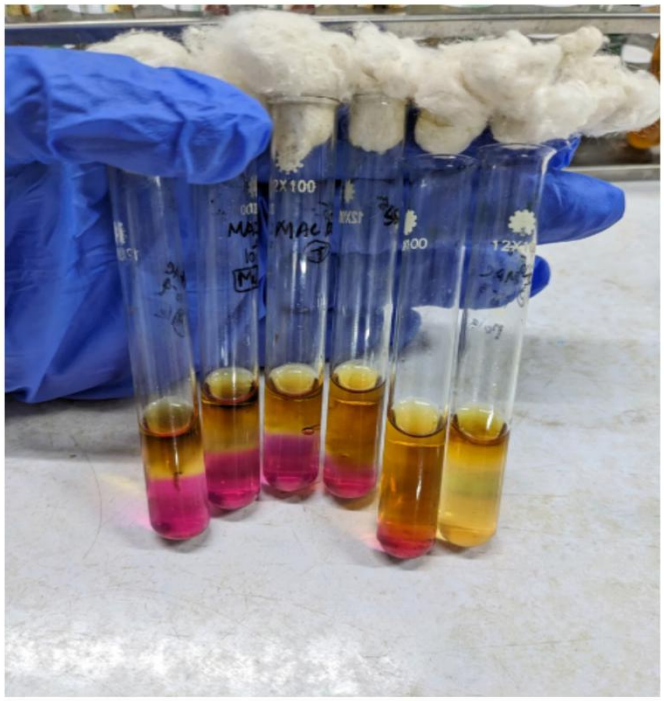
F4S2A	+	+	-	A	A	-	+	-	-	+	***Escherichia coli***
F4S2B	+	+	+	K	A	+	-	-	-	+	***Proteus***

											mirabillis
F4S2C	+	-	-	A	A	-	+	+	-	-	***E coli***
F4S2D	+	+	-	A	A	-	+	+	+	+	***Klebsiella pneumoniae***
F4S2E	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
F4S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
F4S3B	+	+	-	A	A	-	+	+	+	+	***Klebsiella pneumoniae***
F4S3C	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
F4S3D	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***

Isolate ID	**MIU**			**TSI**				**Citrate**	**Oxidase**	**Catalase**	**Presumptive Organism**
	M	**I**	**U**	**Slant**	**Butt**	**H2S**	**Gas**				
NF1S1A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF1S1B	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***
NF1S1C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
NF1S1D	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
NF1S2A	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***

NF1S2B	+	+	-	A	A	-	+	-	-	+	***E coli***
NF1S2C	+	+	-	A	A	-	+	_	-	+	***Klebsiella pneumoniae***
NF1S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF1S3B	+	-	+	A	A	+	-	+	-	+	***Aeromonas hydrophila***
NF1S3C	+	+	-	A	A	-	+	-	-	+	***E coli***
NF2S1A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF2S1B	-	+	-	K	A	-	-	-	-	-	***Shigella spp***
NF2S1C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
NF2S2A	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***
NF2S2B	+	+	-	A	A	-	+	-	-	+	***E coli***
NF2S2C	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
NF2S2D	+	-	-	A	A	-	+	+	-	-	***Enterobacter***
NF2S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF2S3B	-	+	-	K	A	-	-	-	-	-	***Aeromonas hydrophila***
NF2S3C	+	+	-	A	A	-	+	-	-	+	***E coli***
NF3S1A	+	-	-	A	A	+	-	-	-	+	***Proteus mirabilis***
NF3S1B	+	+	-	A	A	-	+	-	-	+	***E coli***

NF3S2A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
NF3S2B	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***
NF3S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF3S3B	-	+	-	K	A	-	-	-	-	-	***Proteus mirabillis***
NF3S3C	+	+	-	A	A	-	+	-	-	+	***E coli***
NF4S1A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
NF4S1B	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
NF4S1C	+	+	-	A	A	-	+	-	-	+	***E coli***
NF4S2A	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
NF4S2B	+	-	-	A	A	+	-	-	-	+	***Salmonella enterica***
NF4S3A	+	+	-	A	A	-	+	-	-	+	***E coli***
NF4S3B	+	+	-	A	A	-	+	-	-	+	***Klebsiella pneumoniae***
NF4S3C	+	+	+	K	A	+	-	-	-	+	***Proteus mirabillis***
NF4S3D	+	+	-	A	A	-	+	+	+	+	***Aeromonas hydrophila***



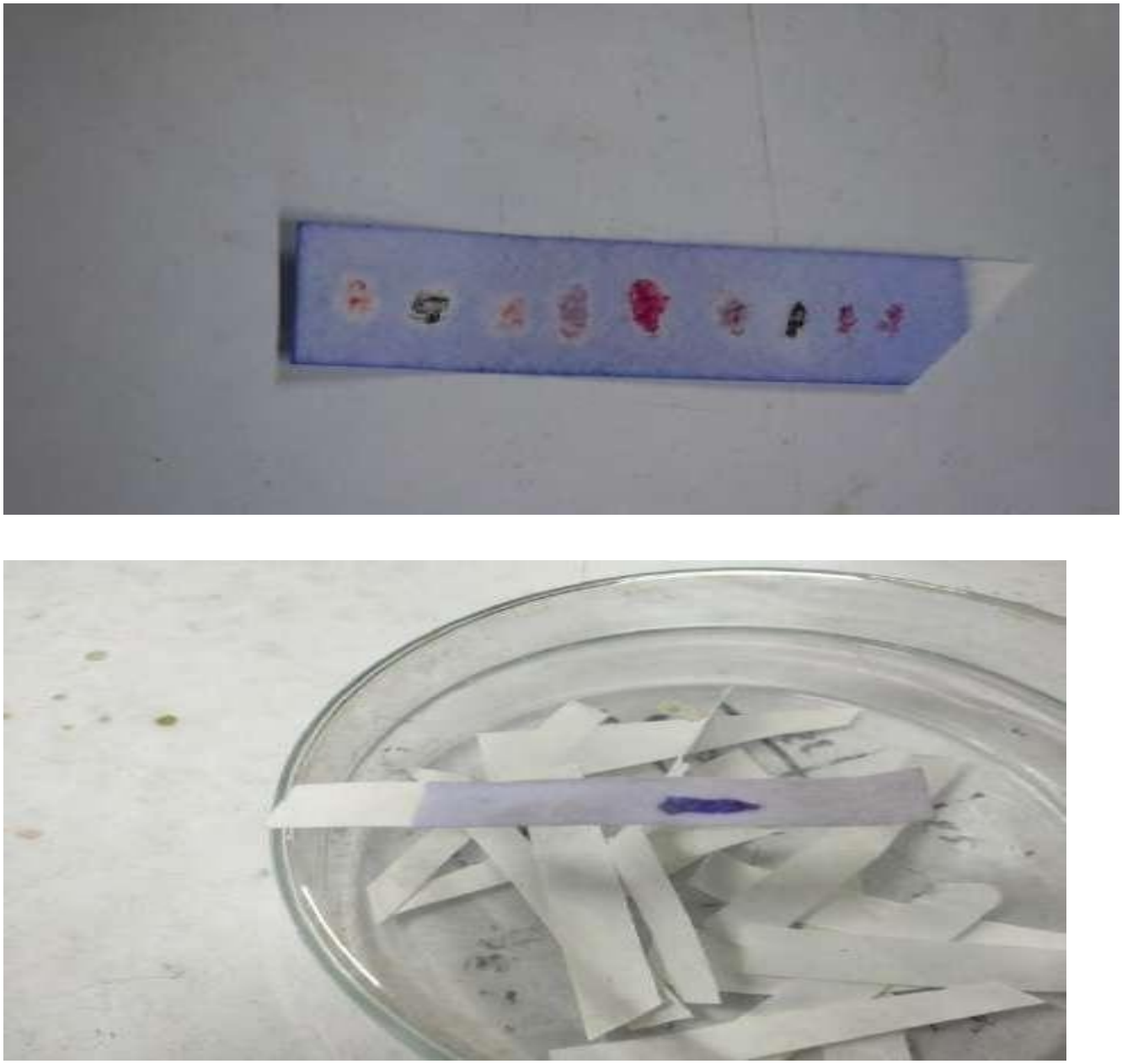


Figure 4.3: Different types of Biochemical Test

4.3 Result of 16S rRNA gene detection

By amplifying and sequencing the 16S rRNA gene, six different bacterial species were detected in samples from integrated fish farms.These were *Escherichia coli, Klebsiella pneumoniae, Proteus mirabillis, W chitiniclastica, Salmonella enterica, and Aeromonas hydrophila.* In contrast,only three bacterial species*: E. coli, K. pneumoniae*, and *P. mirabilis* were identified from non integrated fish farms. The greater diversity observed in integrated

systems may be attributed to higher inputs of organic nutrients and more intricate environmental interactions relative to non-integrated systems.

Table 4.3: Results of 16S rRNA gene detection process

Farms	Isolated ID	Culture Media	Query Coverage %	Resistance to those antibiotics	Organism
Integrated	F2S1B	Mac	100	Te,Cip,pb,Azm,Amp,Fox,E,Fep	***Aeromonas hydrophila***
Integrated	F4S1C	Mac	100	Atm,Te,Pb,Nor,Ctx,Azm,Amp	***Wohlfahrtii monas chitiniclastica***
Integrated	F1S1B	Mac	99	Atm,K,Te,Cip,Pb,Nor,Cn,Amp,Fox,E	***Klebsiella pneumoniae***
Integrated	F2S1D	Mac	100	Atm,K,Te,Nor,Amp,E	***E coli***
Integrated	F1S1E	XLD	99	Atm,k,Te,Pb,Nor,Azm,Amp,Fox	***Salmonella enterica***
Non Integrated	NF1S1A	Mac	99	Atm,K,Te,Pb,E,Amp,Nor,Fep	***E coli***
Non Integrated	NF2S1C	SS	98	Atm,K,Te,Pb,E,Azm,Amp	***Proteus mirabilis***
Non Integrated	NF3S2A	Mac	99	Atm,Te,Nor,Cn,Amp,Fox,E	***Klebsiella pneumoniae***
Non Integrated	NF4S1C	Mac	100	Atm,Te,Pb,Ctx,Amp,E	***E coli***

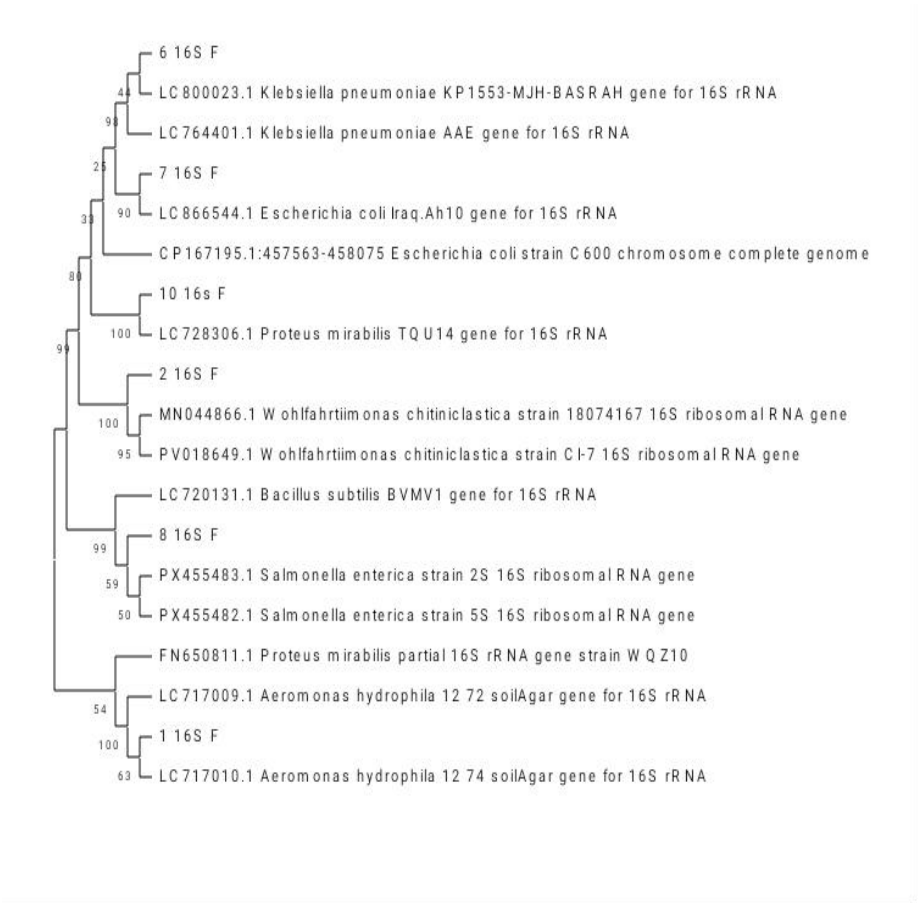


Figure 4.4:The isolates were clearly divided into six main bacterial categories by the phylogenetic analysis of 16S rRNA sequences. *Salmonella enterica, Proteus mirabilis, K. pneumoniae, and E. coli* were shown to cluster together, suggesting their evolutionary link within the Enterobacteriaceae family. With strong bootstrap support (>70%), *E. coli and K. pneumoniae* were identified as closely related sister taxa, suggesting they may share an ancestor and occupy similar ecological niches.There was intra-species variation among these isolates since two sequences from *P.mirabilis* were discovered on different sub-branches.*Wohlfahrtiimonas chitiniclastica's* phylogenetic separation from the other known diseases was confirmed when it was identified as a distinct, well-supported group (99%).*Aeromonas hydrophila*, a genetically more distant lineage usually found in aquatic environments, was categorized independently with complete support (100%).The phylogenetic study concludes by highlighting the microorganisms originating from fish farms' shared and distinct evolutionary relationships.

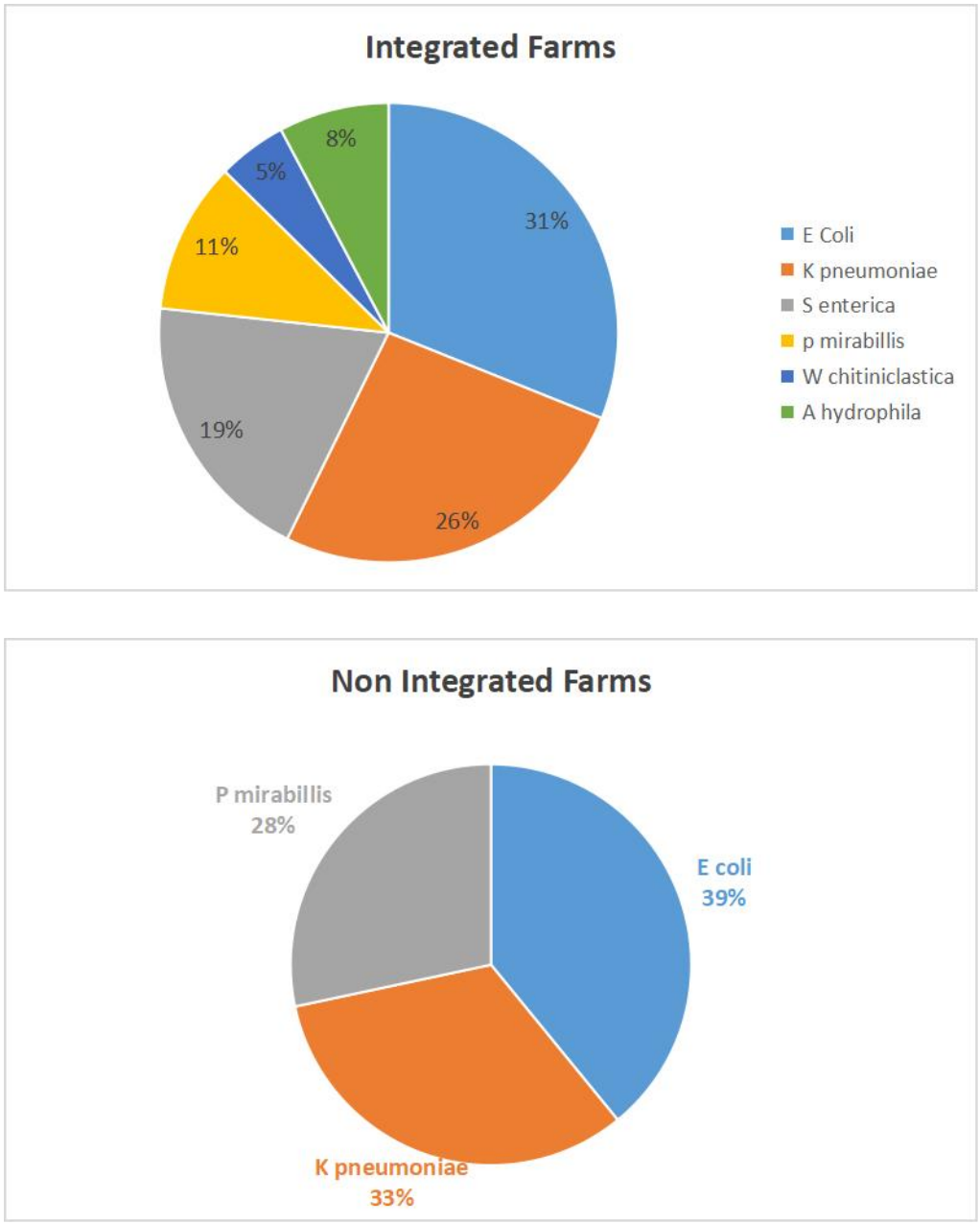


Figure 4.5 :Comparative analysis of total isolated bacteria from two systems

4.4. Antibiotic Sensitivity Test

We have used antibiotic disc of Aztreonam (ATM 30), Kanamycin (K 30), Tetracycline (TE 30), Ciprofloxacin (CIP 5), Polymyxin B (PB 30), Norfloxacin (NOR 30), Gentamycin (CN 10), Cefotaxime (CTX 300), Azithromycin (AZM 15), Ampicillin (AMP 25), Cefoxitin (FOX 30), Sulfamethoxazole-Trimethoprim (SXT25), Erythromycin (E15), Cefepime (FEP 30), Amoxicillin (AMC 30), Colistin (CT 10). According to CLSI guideline 2023, Zone diameters interpretations are given

Table 4.4: Antibiotic susceptibility of 90 isolates from the 24 samples

Antibiogram of 90 isolates

SAMPLE ID		ATM 30	K 30	TE 30	CIP 5	PB 30	NOR 30	CN 10	CTX 300	AZ M 15	A M P 25	FO X 30	SX T 25	E 15
FARM 1	F1S1A	R	R	R	S	S	R	S	I	I	R	S	S	I
	F1S1B	R	R	R	R	R	R	R	S	I	R	R	S	R
	F1S1C	I	R	R	R	R	R	S	S	I	R	S	R	R
	F1S1D	R	S	R	R	S	S	S	S	R	R	R	S	R
	F1S1E	R	R	R	S	R	R	S	I	R	R	R	S	I
	F1S2A	I	R	R	S	I	I	S	S	R	R	S	I	I
	F1S2B	R	R	R	S	R	S	S	S	R	R	R	S	I
	F1S2C	R	R	R	S	S	S	S	I	S	R	R	S	I
	F1S2D	R	I	R	I	S	S	S	S	R	R	S	R	I
	F1S2E	I	R	R	R	S	I	R	S	I	R	S	S	R
FARM 2	F2S1A	S	S	R	S	S	R	S	I	S	I	S	I	R
	F2S1B	S	S	R	R	R	I	S	S	R	R	R	S	R
	F2S1C	S	R	I	S	S	S	I	R	I	I	R	I	I
	F2S1D	R	R	R	I	S	R	I	S	S	R	S	S	R
	F2S1E	R	S	R	S	I	S	S	S	R	I	R	R	I
	F2S2A	I	I	R	S	R	I	S	R	I	R	S	S	R
	F2S2B	I	R	I	R	S	S	R	I	S	R	S	S	I
	F2S2C	I	S	R	S	S	I	S	S	I	R	S	S	I

	F2S2D	**I**	**R**	**R**	**I**	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**S**	**S**
	F2S2E	**R**	**R**	**R**	**S**	**I**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**I**
FARM 3	**F3S1A**	**R**	**R**	**R**	**S**	**S**	**I**	**S**	**S**	**R**	**I**	**R**	**S**	**I**
	F3S1B	**I**	**R**	**I**	**S**	**S**	**R**	**S**	**I**	**I**	**R**	**S**	**I**	**R**
	F3S1C	**S**	**S**	**I**	**S**	**I**	**S**	**S**	**I**	**R**	**R**	**I**	**R**	**R**
	F3S1D	**S**	**S**	**R**	**R**	**S**	**I**	**R**	**S**	**I**	**I**	**S**	**S**	**R**
	F3S1E	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**I**
	F3S2A	**S**	**I**	**S**	**I**	**R**	**I**	**S**	**S**	**R**	**S**	**R**	**I**	**S**
	F3S2B	**R**	**S**	**S**	**S**	**R**	**S**	**I**	**R**	**I**	**R**	**S**	**I**	**R**
	F3S2C	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
	F3S2D	**I**	**R**	**R**	**S**	**R**	**R**	**S**	**S**	**I**	**I**	**S**	**S**	**I**
	F3S2E	**S**	**R**	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**R**	**I**	**R**	**R**
FARM 4	**F4S1A**	**S**	**I**	**R**	**S**	**I**	**S**	**S**	**S**	**R**	**R**	**I**	**R**	**R**
	F4S1B	**R**	**R**	**R**	**S**	**R**	**I**	**S**	**S**	**R**	**R**	**I**	**S**	**R**
	F4S1C	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**I**
	F4S1D	**I**	**S**	**R**	**S**	**R**	**I**	**S**	**S**	**S**	**R**	**S**	**S**	**R**
	F4S1E	**R**	**S**	**R**	**S**	**R**	**S**	**S**	**I**	**R**	**R**	**R**	**S**	**I**
	F4S2A	**R**	**I**	**R**	**S**	**R**	**I**	**S**	**S**	**I**	**I**	**I**	**R**	**I**
	F4S2B	**I**	**I**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**
	F4S2C	**S**	**R**	**I**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
	F4S2D	**R**	**R**	**R**	**S**	**I**	**I**	**S**	**S**	**I**	**R**	**S**	**S**	**R**
	F4S2E	**I**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**I**	**R**	**S**	**S**	**S**

Non Integrated Farm 1	**NF1S1A**	**R**	**R**	**R**	**S**	**R**	**R**	**I**	**S**	**I**	**R**	**I**	**S**	**R**
	NF1S1B	**S**	**S**	**R**	**I**	**R**	**R**	**R**	**R**	**I**	**R**	**S**	**S**	**S**
	NF1S1C	**I**	**I**	**I**	**I**	**R**	**I**	**S**	**I**	**R**	**S**	**S**	**S**	**S**
	NF1S1D	**S**	**R**	**R**	**S**	**R**	**I**	**S**	**I**	**I**	**S**	**S**	**S**	**S**
	NF1S2A	**S**	**I**	**I**	**I**	**R**	**R**	**S**	**S**	**S**	**I**	**S**	**S**	**S**
	NF1S2B	**I**	**S**	**R**	**R**	**R**	**R**	**S**	**I**	**S**	**I**	**S**	**S**	**S**
	NF1S2C	**S**	**I**	**R**	**R**	**R**	**R**	**R**	**I**	**I**	**S**	**I**	**S**	**R**
	NF1S3A	**I**	**I**	**R**	**R**	**I**	**I**	**R**	**R**	**R**	**R**	**I**	**R**	**R**
	NF1S3B	**R**	**I**	**R**	**I**	**R**	**R**	**I**	**R**	**I**	**S**	**I**	**I**	**S**
	NF1S3C	**I**	**I**	**R**	**R**	**R**	**I**	**I**	**I**	**R**	**S**	**I**	**I**	**S**
Non Integrated Farm 2	**NF2S1A**	**l**	**R**	**S**	**R**	**R**	**R**	**I**	**I**	**R**	**I**	**I**	**I**	**S**
	NF2S1B	**R**	**I**	**R**	**I**	**I**	**R**	**I**	**I**	**R**	**I**	**R**	**I**	**I**
	NF2S1C	**R**	**R**	**R**	**I**	**R**	**I**	**I**	**I**	**R**	**R**	**I**	**S**	**R**

	NF2S2A	**I**	**I**	**R**	**I**	**R**	**I**	**I**	**I**	**R**	**I**	**R**	**S**	**I**
	NF2S2B	**R**	**I**	**I**	**R**	**I**	**I**	**I**	**I**	**S**	**I**	**I**	**S**	**I**
	NF2S2C	**R**	**I**	**I**	**I**	**R**	**R**	**I**	**I**	**S**	**S**	**I**	**S**	**I**
	NF2S3A	**I**	**R**	**R**	**I**	**I**	**I**	**R**	**R**	**S**	**I**	**R**	**I**	**S**
	NF2S3B	**I**	**R**	**R**	**I**	**I**	**I**	**I**	**R**	**I**	**R**	**I**	**I**	**I**
	NF2S3C	**R**	**R**	**I**	**R**	**I**	**R**	**I**	**I**	**S**	**R**	**I**	**I**	**I**
	NF2S3D	**I**	**R**	**R**	**I**	**I**	**I**	**S**	**I**	**I**	**I**	**R**	**R**	**I**
Non Integrated Farm 3	**NF3S1A**	**I**	**I**	**R**	**I**	**R**	**I**	**R**	**I**	**I**	**R**	**R**	**R**	**I**
	NF3S1B	**I**	**I**	**I**	**R**	**I**	**S**	**I**	**I**	**S**	**R**	**R**	**R**	**I**
	NF3S2A	**R**	**I**	**R**	**I**	**I**	**I**	**R**	**R**	**I**	**R**	**R**	**R**	**I**
	NF3S2B	**I**	**R**	**R**	**R**	**I**	**I**	**R**	**I**	**I**	**R**	**I**	**R**	**R**
	NF3S3A	**S**	**I**	**I**	**R**	**I**	**I**	**R**	**I**	**S**	**R**	**I**	**I**	**S**
	NF3S3B	**R**	**R**	**I**	**I**	**I**	**R**	**S**	**I**	**S**	**R**	**I**	**I**	**I**

	NF3S3C	**R**	**R**	**R**	**I**	**I**	**R**	**S**	**S**	**S**	**R**	**I**	**I**	**I**
Non Integrated Farm 4	**NF4S1A**	**I**	**R**	**R**	**I**	**I**	**R**	**S**	**S**	**I**	**R**	**I**	**I**	**R**
	NF4S1B	**R**	**R**	**I**	**I**	**R**	**I**	**S**	**S**	**I**	**I**	**R**	**I**	**R**
	NF4S1C	**R**	**I**	**R**	**I**	**R**	**I**	**I**	**R**	**I**	**R**	**I**	**I**	**R**
	NF4S2A	**R**	**I**	**I**	**R**	**R**	**I**	**I**	**R**	**R**	**I**	**I**	**I**	**I**
	NF4S2B	**I**	**I**	**R**	**R**	**R**	**R**	**I**	**S**	**R**	**I**	**I**	**R**	**I**
	NF4S3A	**I**	**R**	**R**	**R**	**I**	**I**	**R**	**S**	**I**	**R**	**I**	**R**	**R**
	NF4S3B	**I**	**I**	**R**	**R**	**R**	**I**	**I**	**I**	**S**	**R**	**R**	**R**	**I**
	NF4S3C	**R**	**I**	**I**	**R**	**R**	**I**	**R**	**R**	**S**	**R**	**I**	**I**	**R**
	NF4S3D	**I**	**R**	**R**	**I**	**I**	**R**	**I**	**I**	**I**	**R**	**R**	**I**	**I**

Here, S=Sensitive, I=Intermediate and R= Resistant

Figure 4.6 : Overall Antibiotic Susceptibility Test Result of Both Farms

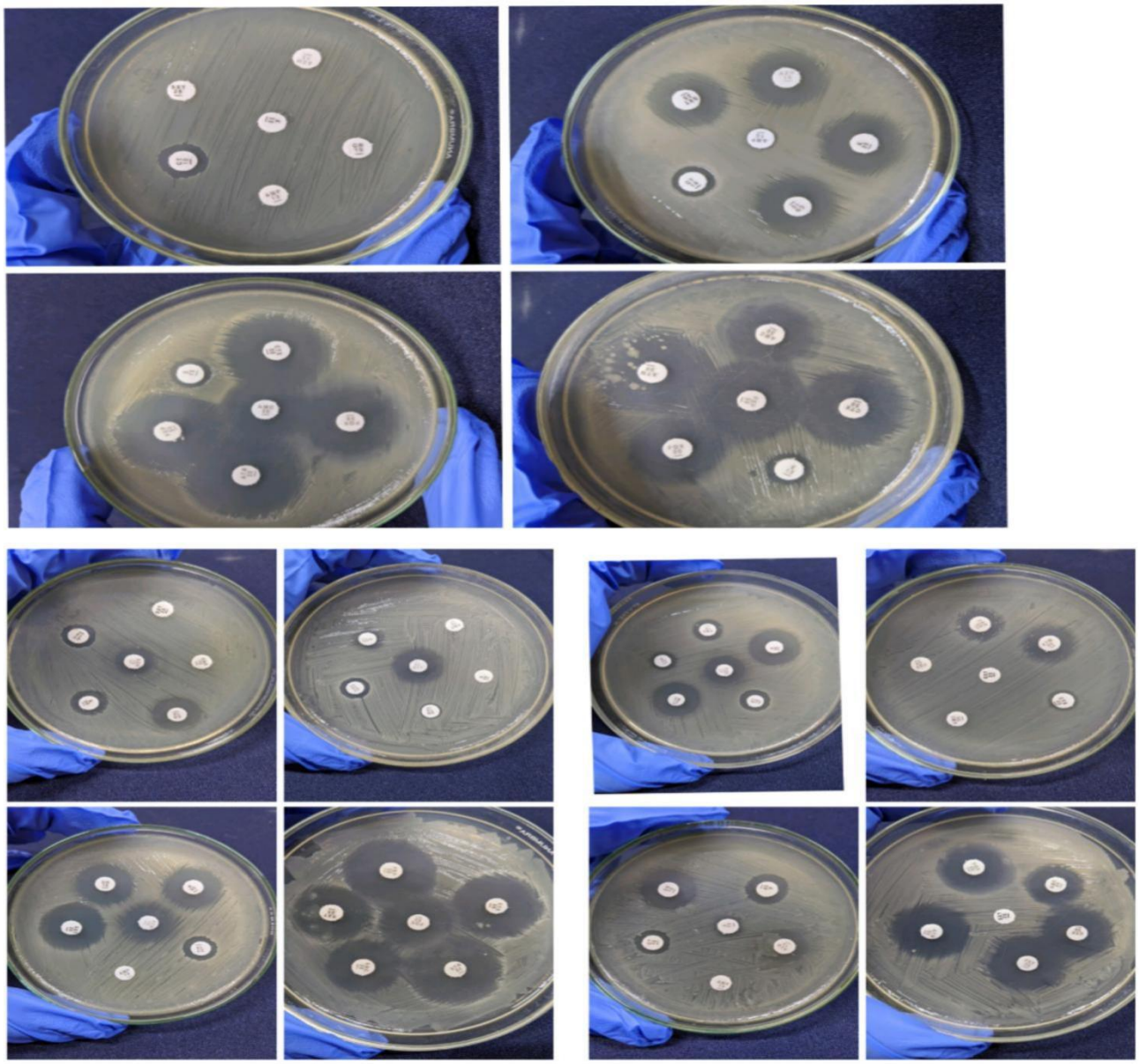


Figure 4.7: Patterns of Antibiotic Susceptibility Zone in Both Cases

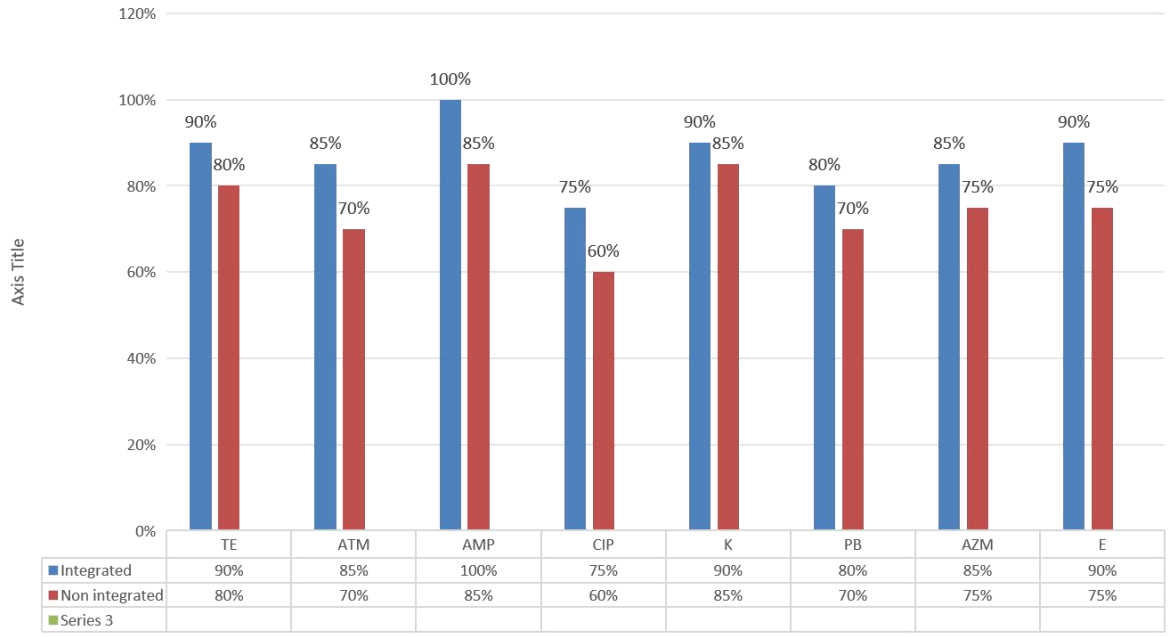


Figure 4.8: Overall Antibiotic Prevalence Rates in Two Farms.In integrated farms ampicillin,tetracycline,kanamycin,erythromycin showed highest resistance rate of 100% followed by 90% and 85%,where non integrated farms exhibited lower resistance rates between 60 to 70% of those antibiotics.

4.5. Detection of Antibiotic Resistance Genes

Table 4.5: The results of the detection of major group of antibiotic resistance genes

Farm No	Isolate ID	*blaCTX-M*	*blaTEM*	*tetA*	*sul1*	*qnr*
1	F1S1B	+	-	+	-	+
	F1S1D	+	+	+	-	-
	F1S2B	-	-	-	+	-
	F1S3B	+	+	-	+	+

2	F2S1D		-	+	-	-
	F2S1E	-	-	-	+	-
	F2S2D	-	-	-	-	+
	F2S3C	+	-	-	-	+
3	F3S1A	-	+	+	-	-
	F3S1D	-	-	-	+	+
	F3S2C	-	-	-	-	-
	F3S3A	-	+	+	-	-
4	F4S1B	-	-	+	-	-
	F4S1C	+	-	-	+	-
	F4S2B	-	-	+	-	-
	F4S3C	+	-	-	+	-
	F4S3D	-	-	+	-	-
1(Non Integrated)	NF1S1A	-	-	+	-	-
	NF1S2B	-	-	-	-	-
	NF1S3A	-	-	-	-	-
2(Non Integrated)	NF2S1C	+	-	+	-	-
	NF2S1B	-	-	-	+	+
	NF2S3B	+	+	+	+	-
3(Non Integrated)	NF3S1A	-	-	-	-	-

	NF3S2A	-	-	-	-	-
	NF3S2B	-	-	-	-	-
4(Non Integrated)	NF4S1C	+	-	-	-	+
	NF4S2B	-	-	-	+	+
	NF4S3B	-	-	-	-	-
	NF4S3C	-	+	-	-	-

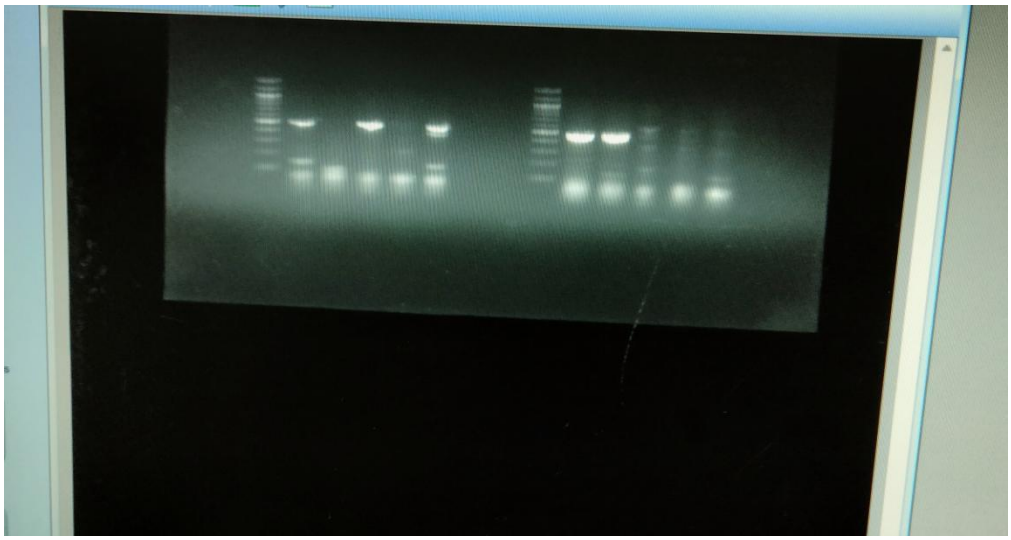


Figure 4.9: *blaCTX-M* resistance gene detection. Primer bp 650, Amplicons were detected in 500 bp.

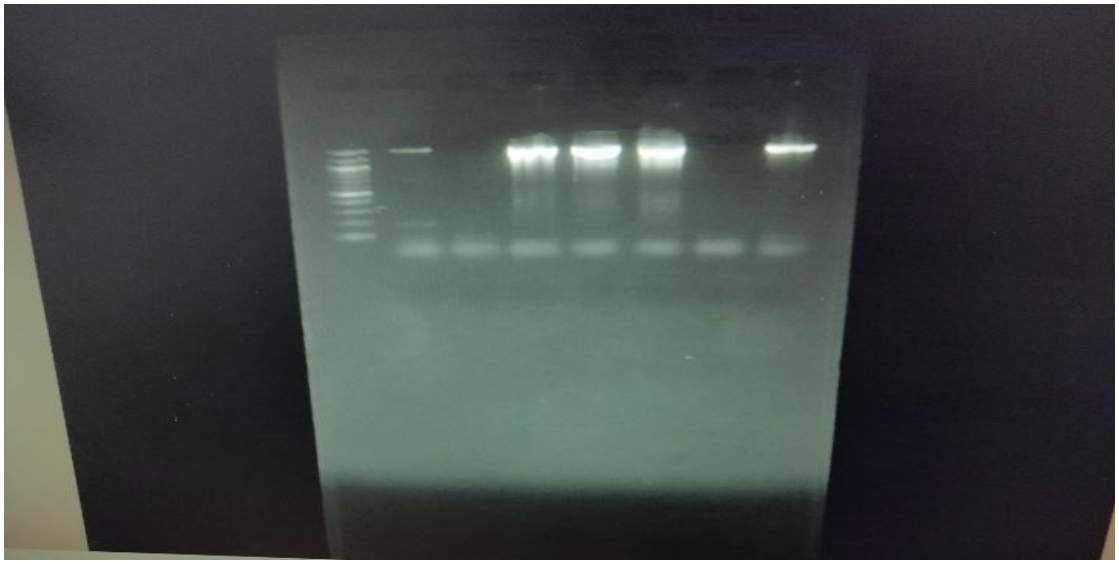


Figure 4.10: *blaTEM* gene detection. Primer base pair 900 bp, amplicons were detected near about the length.

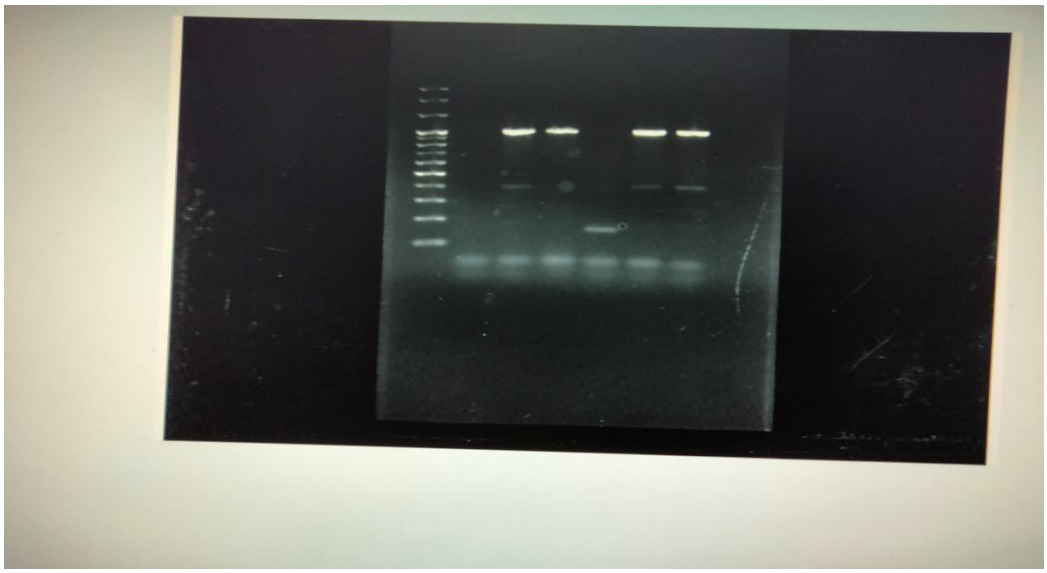


Figure 4.11 : *tetA* gene detection. Primer size 950 bp,amplicons were detected in 900 bp

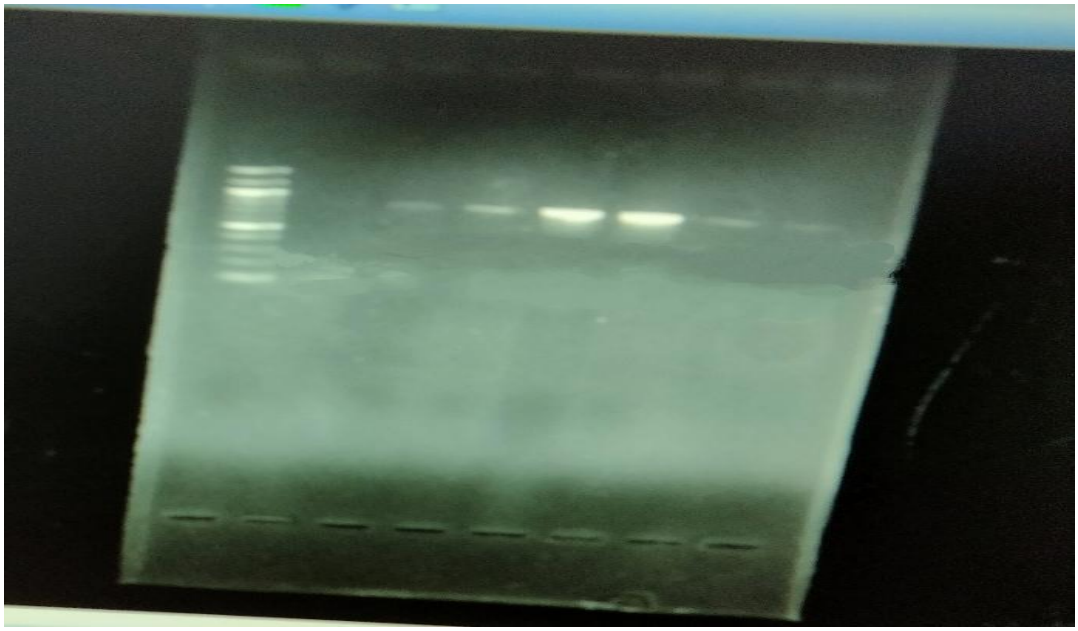


Figure 4.12: *sul1* gene detection, primer size 800 bp,amplicons were detected between 700 and 800 bp.

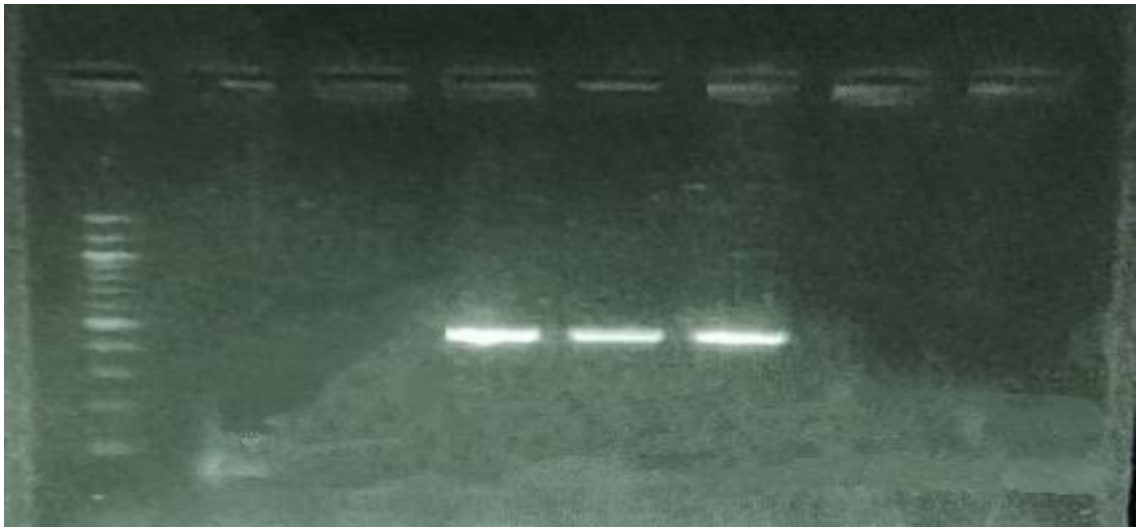


Figure 4.13:qnr gene detection, primer size 700 bp,amplicons were detected between 400 and 500 bp.

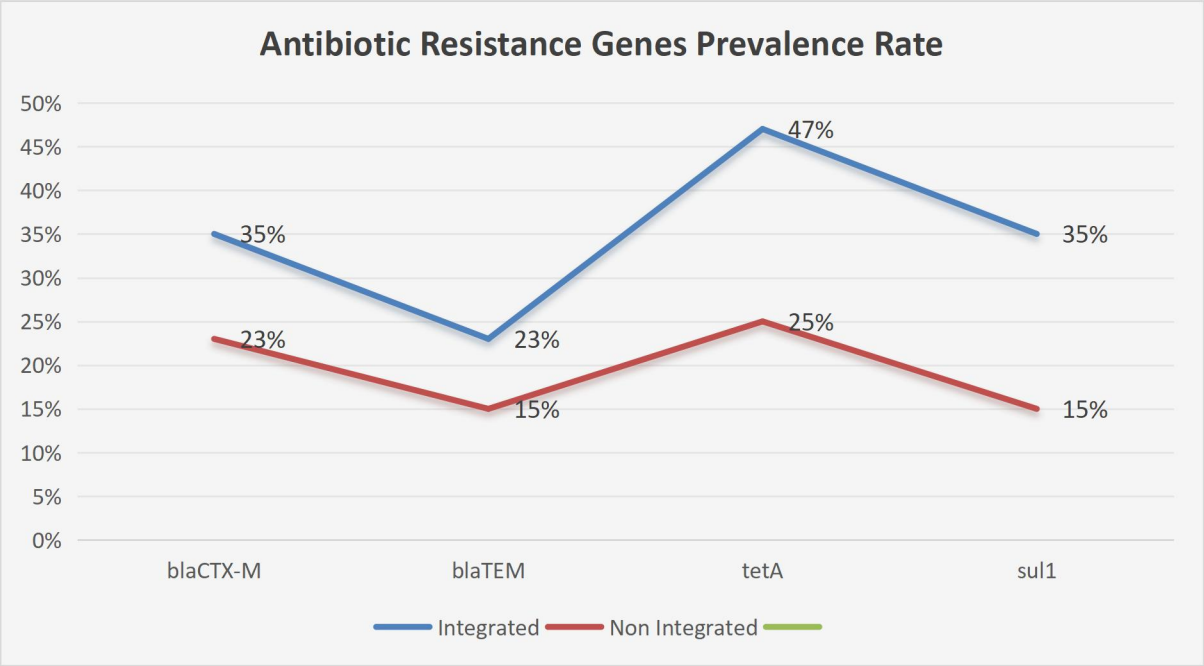


Figure 4.14:Major Antibiotic Resistance Genes Detection Rates in Both Cases

Chapter-5: Discussion and Conclusion

5.1 Discussion

This study uncovered a worrying yet frequently seen pattern,the existence of various Gram-negative bacterial species in aquaculture fish farms. A total of 90 bacterial isolates were obtained, which consisted of seven species (*Aeromonas hydrophila, Wohlfahrtiimonas chitiniclastica, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae, and Proteus mirabilis)* from the sampled fish.

In the case of integrated farming systems, E. coli (31%) was the most frequently isolated bacterium, followed by *K. pneumoniae* (26%), *S. enterica* (19%), *P. mirabilis* (11%), A. *hydrophila* (8%), and *W. chitiniclastica* (5%).On the other hand, non-integrated farms exhibited a greater occurrence of *E. coli* (42%), with *K. pneumoniae (*30%) and *P. mirabilis* (28%), highlighting a significant differences in bacterial distribution between the two types of farming systems.Additionally, 16S rRNA sequencing was performed to corroborate the identification of these strains, increasing the chances that the observed resistance patterns match the identified taxa.

The current findings of this study show that microorganisms from integrated and non-integrated fish farms in Bangladesh exhibit distinctly different patterns of antibiotic resistance.Multi-drug resistance (MDR) bacterial strains are typically more common on integrated farms because in this case aquaculture coexists with livestock or poultry [60–62].This finding supports earlier worries about the spread of resistant bacteria and animal waste residues into aquatic environments. An ideal selection pressure that promotes the emergence and maintenance of resistance characteristics in bacteria appears to be produced by the continuous flow of antibiotics through feed, animal waste, and water [65–68].

On the other side, isolates from non-integrated farms demonstrated significantly lower levels of resistance, suggesting that less exposure to trash containing antibiotics reduces selective pressure.But, it is important to recognize that resistance was still found in non-integrated systems to some extent.This suggests that antibiotic resistance is a major concern in aquaculture of Bangladesh rather than only one that affects integrated systems [70].The establishment and dissemination of resistant genes in both farming systems may also be influenced by factors like improper antibiotics use, availability to over-the-counter pharmaceuticals, lack of withdrawal periods and low farmer awareness, education as well. [80–82].

The resistance to commonly used antibiotics such as tetracycline, ampicillin, ciprofloxacin, amoxicillin, and kanamycin was one of the study's most startling discoveries.Testing for antibiotic susceptibility revealed different patterns of resistance in *Salmonella enterica, Klebsiella pneumoniae, and E. coli*.At 100 %, ampicillin and 90% tetracycline had the highest rates of resistance,with amoxicillin coming in second at 75%. Ciprofloxacin resistance was found to be moderate at 50%.On the other hand, just two antibiotics cefoxitin (fox) and sulfamethoxazole-trimethoprim (sxt) showed the highest sensitivity, indicating a comparatively better efficacy.

The incidence of drug resistance may be explained by the fact that these treatments are frequently used in fish farms as preventative measures and to treat bacterial illnesses [70].Additionally, 47%, 35%, 29%, and 23% of isolates from integrated farms had significant resistance genes such *tetA, blaCTX-M, sul1, and blaTEM,* whereas the comparable percentages in non-integrated farms were 25%, 23%, and 15%.These findings are consistent with the collected data from Bangladesh and the surrounding region that aquaculture settings serve as reservoirs for resistant bacteria and also the resistance genes that are linked to them [62–64].

Bacteria have the ability to progressively adapt, modify, and spread resistance genes through plasmids and horizontal gene transfer mechanisms when antibiotics are continuously provided randomly at sub-therapeutic dosages [82].This increased prevalence rate of MDR isolates in integrated farms was probably caused by this mechanism.

The same bacterial taxa and resistance genes from Bangladeshi seafood and aquaculture have been found in recent studies.For instance, numerous studies evaluating pond sediments, coastal seafood, and cultured fish have consistently identified *E. coli, Salmonella, Klebsiella, and Pseudomonas* while noting notable levels of resistance to commonly used antibiotics like ampicillin, tetracyclines, and fluoroquinolones [60–70].While *tetA* and *sul*1 are among the most commonly reported antibiotic resistance genes in aquatic environments, research conducted in Bangladesh and neighboring regions has consistently detected *blaTEM* and *blaCTX-M* from ESBL-producing Enterobacteriaceae in fish or seafood specimens [81–90].These investigations imply that our results are part of a broader national issue related to environmental routes and antibiotic consumption practices.

The sampling context (whether from pond water, fish tissue, or market seafood), seasonal influences farming practices (integrated versus non-integrated) and the kinds of antibiotics

used locally can frequently account for variations in our results, such as the frequency of one gene versus another [75–78].For example, a number of studies show that ESBL genes are more common in systems affected by human or animal waste, which is consistent with the higher multidrug-resistant burden observed for integrated farms in our earlier investigations [84–85].?

In isolates, the co-occurrence of *blaCTX-M/blaTEM* with *tetA* and *sul1* is both mechanistically problematic and physiologically feasible.These genes are often found on mobile genetic elements that facilitate horizontal gene transfer across bacterial species found in water and soil, such as plasmids, transposons, and class 1 integrons [72–74].Since sul1 is usually found in the conserved area of class 1 integrons, its presence is particularly suggestive of activity related to these integrons and its identification frequently implies a genetic platform that can host several resistance cassettes [81].The persistence and spread of these mobile elements are encouraged by selection pressure created by the regular or sub-therapeutic use of antibiotics in aquaculture (via medicated feed, livestock runoff, or prophylactic treatments) [68].As a result, the gene combinations we found align with well-established concepts of how antibiotic resistance genes spread in aquatic environments.

The implications for public health are substantial from a One-Health standpoint. Fish that contain multidrug-resistant (MDR) or ESBL-producing bacteria can be consumed by humans. These bacteria can then be discharged into the environment through waste, sediment, or water, creating pathways for the transfer of antibiotic resistance genes (ARGs) to human infections [60–65].In fact, recent research from Bangladesh has shown that seafood is tainted with ESBL-producing E. coli; for example, raw seafood from coastal markets has been shown to have *blaTEM* and *blaCTX-M* [76].Furthermore, research in non-aquatic industries like poultry shows that comparable ESBL genes (*blaTEM, blaSHV,* etc.) are highly prevalent throughout Enterobacteriaceae, indicating that ARGs are common in all animal-rearing settings [76–77].

Therefore, our results imply that aquaculture should be recognized as an important link in the chain of ARG transmission, connecting humans, livestock, aquatic species, and the environment.As evidenced by earlier reports of plasmid-mediated transfer among fish, water, and workers in integrated farming operations [70–80], these systems may further promote the spread of ARGs because integrated farms—which can combine livestock, poultry, and agricultural runoff typically use more antibiotics.

As a result, mitigation efforts should focus on water quality, overall aquaculture management, and upstream sources (such livestock farms and waste management) in addition to aquaculture methods [80–83].Reducing needless antibiotic use, enforcing withdrawal periods, enhancing biosecurity, and implementing waste management programs are all critical.Additionally, tracking the mobility of ARGs and detecting high-risk mobile genetic elements might benefit from molecular surveillance techniques (such as plasmid type and whole-genome sequencing) [80–90].

The identification of several Gram-negative bacteria in aquaculture fish that carry clinically significant ARGs highlights the urgent need for prudent management and coordinated surveillance.Without action, aquaculture's contribution to the worldwide problem of antibiotic resistance could worsen, endangering the health of both humans and animals.

5.2 Conclusion

This study unequivocally shows that aquaculture in Bangladesh, particularly in integrated agricultural systems, serves as a major source of clinically important antibiotic resistance genes and Gram-negative bacteria.The impact of animal waste, antibiotic use, and environmental transmission on resistance patterns is shown in the higher frequency of multi-drug resistant bacteria in integrated farms.Although antibiotic-resistant bacteria were present in non-integrated systems despite their lower resistance levels, this suggests that antimicrobial resistance in aquaculture is a pervasive and growing problem rather than being limited to a single farming technique.The continuous selective pressure found in fish-rearing environments is demonstrated by the frequent detection of resistance to widely used antibiotics, especially tetracycline, ampicillin, amoxicillin, and kanamycin, as well as the identification of critical resistance genes like tetA, blaCTX-M, sul1, and blaTEM.These resistance genes' coexistence on mobile genetic elements is concerning since it promotes quick horizontal gene transfer across bacterial species, accelerating the spread of resistance both inside and outside of aquatic ecosystems.These findings support the One-Health strategy by showing that antibiotic resistance can spread from farms to animals, the environment, and ultimately to people through food intake and environmental exposure. The danger of resistant germs infiltrating the human population is growing as aquaculture expands to meet food

security demands.Quick action is necessary to safeguard aquaculture's sustainability as well as public health.Emphasis should be placed on the responsible use of antibiotics, strict drug administration regulations, efficient waste management, farmer education, and regular molecular monitoring.Improving these tactics can help prevent resistance from spreading while maintaining aquaculture operations' effectiveness.In conclusion, this work adds to the growing body of evidence showing aquaculture plays a critical role in the larger picture of antimicrobial resistance.In aquaculture environments, resistant bacteria and the genes they carry may endure and spread without treatment, posing a long-term threat to human welfare, environmental stability, and animal health.

Chapter-6: References

6.References

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Appendix

APPENDIX-I

1.Trypticase soy broth medium (TSB) (pH 7.3 ± 0.2)

Components	Quantity in gram
Pancreatic digest of casein	17
Papaic digest of soybean meal	03
Sodium Chloride	05
Dextrose (Glucose	2.5
Dipotassium hydrogen phosphate	2.5
Agar	15

2.Luria Bertani broth medium (LB)(pH 7.00 ± 0.2)

Components	Quantity in gram
Tryptone	10
Yeast extract	05
Sodium Chloride (NaCl)	05
Distilled Water	1000 ml

3. MacConkey Agar

Ingredients	Amount (gram/litre)
Peptone (Pancreatic digest of gelatin)	17
Proteose peptone (meat and casein)	03

Lactose monohydrate	10
Bile salts	1.5
Sodium Chloride	05
Neutral red	0.03
Crystal Violet	0.001
Agar	13.5
Distilled Water	1 L
Final pH	7.1

4. Shigella Salmonella Agar(SS) : pH (at 25°C) 7.0 ± 0.2

Ingredients	Amounts (gram/litre)
Beef Extract	05
Enzymatic Digest of Casein	2.5
Enzymatic Digest of Animal Tissue	2.5
Lactose	10.00
Bile Salts	8.50
Sodium Citrate	8.50
Sodium Thiosulfate	8.50
Ferric Citrate	1.00
Brilliant Green	0.00033
Neutral red	0.025

Agar	13.50
Distilled Water	1 L

5.XLD Agar:Final pH 7.4 ± 0.2

Components	Amounts (gram/litre)
Xylose	3.75
L-Lysine	05
Lactose	7.5
Sucrose	7.5
Yeast Extract	03
Sodium Deoxycholate	2.5
Sodium Thiosulfate	6.8
Ferric Ammonium Citrate	0.8
Phenol Red	0.08
Agar	15
Distilled Water	1L

6.Mueller Hinton Agar (MHA):Final pH 7.3 ± 0.1 at 25ºC

Ingredients	Amounts (gram/litre)
Beef Extract	2.00
Acid Hydrolysate of Casein	17.50
Starch	1.50

Agar	17.00
Distilled Water	1 L

APPENDIX-II

1. Triple Sugar Iron (TSI) Agar (Final PH at 25°C: 7.3+/-0.2)

2. Urea Agar (Final PH at 25°C: 6.8+/-O.2)

Ingredients	Gm/liter
Urea	20.00
Na2HPO4	1.5
KH2HPO4	5.00
Yeast Extract	2.00
Phenol Red	0.01
Distilled Water	1000 ml

3. Kovacs Reagent

Ingredients	Amounts
p-Dimethylamino-benzaldehyde	05
Amyl Alcohol (normal only)	75
HCL (concentrated)	25
Distilled Water	Up to 1 liter

4.Simmons Citrate Agar (Final pH 6.9 +/- 0.2 at 25 degrees C.)

Ingredients	Amounts
Sodium Chloride	05 gm
Sodium Citrate	02 gm
Ammonium Dihydrogen Phosphate	01gm
Dipotassium Phosphate	01gm
Magnesium Sulfate (heptahydrate)	0.2 gm
Bromothymol Blue	0.08 gm
Agar	15 gm
Deionized Water	1000 gm
PH	6.9 +/- 0.2 at 25 degrees C

5. Crystal Violet:

Amount	Ingredients
Crystal Violet	02 g
95% ethyl alcohol	20 ml
Ammonium citrate monohydrate	0.8 g
Distilled Water	80 ml

6. Methyl Red:

Ingredients	gm/litre
Methyl Red	0.2
Ethyl Alcohol	60

Distilled Water	40 ml

7.Composition of a 2X PCR Mastermix

Component	Purpose
Taq DNA Polymerase	Enzymes for DNA synthesis
dNTPs	DNA building blocks
MgCL2	Essential cofactors for taq
Reaction Buffer	Maintains optimal pH
Tracking Dye	Allows direct gel loading
Loading Buffer	For electrophoresis

APPENDIX III

Apparatus Used

Instrument	Manufacturer
Autoclave (Model 111,-42E)	Tokyo, Japan
Centrifuge machine	Sigma,USA
Class-11 Al biological safety cabinet	Thermo Forma, USA
Duran Bottle	Scott Germany
Electric balance model no 2 1 OS	Sartorius, Germany
Eppendrof tubes (I, 5m)	Eppendrof, Germany.
Freezer (-80°C)	Thermo Forma, USA
Freeze 4°C	West frost
Fridge 8°C model no. M1R-253	Japan

Incubator, WTB binder, model no. D-78502	Germany
Glassware	Pyrex brand, USA
Magnetic stirrer	Coming, UK
Microcentrifuge, Eppendrof centrifuge.	Germany
Micropipettes	Eppendrof, Germany
Micropipette tips	Labsystems, Finland
Microwave oven, model no. CE2933N	Samsung, Korea
PCR Thermo cycler	Germany
Power supply	BIO-RAD, USA.
Sterilizer	Memmert, Germany
Vortex Mixer	Fisher Brand, England
Autoclave, model WAC-80	Daihan, korea
Laminar air flow	Esco, Singapore
Incubator, Model LSIS-B2V IC55	MMM group, Germany
Micropipette	Glassco,UK
Refrigerator (4°C), model W2D-A90	Walton, Bangladesh
Dry heat sterilizer, Model LSIS — B2V/EC55	MMM group, Germany
Vortex Mixer, Model VM-1000	Digsystem Laboratory Instruments Inc. Taiwan
Water bath	England
Class II Microbiological safety cabinet	Thermo Fisher Scientific, USA
Heat block	Burnstead International