PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN SELECTED BACTERIAL ISOLATES FROM *PENAEUS VANNAMEI*

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of the requirements
for the degree of

M.F.Sc. (Aquatic Animal Health Management)

by

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Dedicated to the Almighty God

&

My beloved Family

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Dated: 30th August 2020

CERTIFICATE

Certified that the dissertation entitled **"PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN SELECTED BACTERIAL ISOLATES FROM *PENAEUS VANNAMEI*"** is a bonafide record of independent research work carried out by **Ms. Bathina. Prathibha** during the period of study from August, 2019 to August, 2020 under our supervision and guidance for the degree of **Master of Fisheries Science (Aquatic Animal Health Management)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

रोगजनकों का रोगाणुरोधी प्रतिरोध (एएमआर) वैश्विक जन स्वास्थ्य के लिए एक गंभीर खतरा है । जलकृषि में एंटीबायोटिक दवाओं का अनियमित उपयोग जलीय वातावरण से अलग एंटीमाइक्रोबियल प्रतिरोधी बैक्टीरिया के उद्भव और प्रसार के कारणों में से एक है जो जलीय जीवों और मानव स्वास्थ्य के लिए खतरा पैदा करता है । रोगनिरोधी और विकास प्रवर्तकों के रूप में एंटीबायोटिक दवाओं के व्यापक उपयोग के कारण एम्ब्रमीडिंग एएमआर प्रसार में महत्वपूर्ण भूमिका निभाता है। वर्तमान अध्ययन में महाराष्ट्र और गुजरात के सुसंस्कृत झींगा से *एस्चेरिचिया कोलाई, स्टेफिलोकोकस ऑरियस* और *विब्रियो एसपीपी* को अलग-थलग करने और उनकी विशेषता बनाने और इन चुनिंदा बैक्टीरिया से जुड़े रोगाणुरोधी प्रतिरोध के फेनोटाइपिक और जीनोमिक लक्षणों को निर्धारित करने का प्रस्ताव किया गया था । कुल ४०३ बैक्टीरियल आइसोले को अलग-थलग कर 26 विभिन्न फार्मों से पहचाना गया । 403 जीवाणु आइसोले में से *एस्चेरिचिया. कोलाई* (113), *स्टेफिलोकोकस. ऑरियस* (62) और सुक्रोज निगेटिव *विब्रियो एसपीपी* (138) को अलग-थलग किया गया और विभिन्न कृषि स्रोतों के 156 झींगा नमूनों से विशेषता। डिस्क प्रसार विधि का उपयोग करके विभिन्न एंटीबायोटिक दवाओं के खिलाफ आइसोलेट का एंटीमाइक्रोबियल संवेदनशीलता पैटर्न किया गया था। अलग-थलग पड़े तीन अलग-अलग बैक्टीरिया में से, प्रतिरोध की एक उच्च व्यापकता एंटीबायोटिक दवाओं के ए-लैक्टाम वर्ग के खिलाफ थी, और 134 आइसोले ने अज़्ट्रेनम और सेफोटैक्सिम के साथ एम्पिसिलिन के खिलाफ प्रतिरोध प्रदर्शित किया। कोई भी आइसोलेट Trimethoprim/Sulfamethoxazole, क्लोरम्फेनिकोल, और Amoxicillin/क्लावुलानिक एसिड के लिए प्रतिरोधी पाया गया । *ई. कोलाई* आइसोलेट का 28.5%, एस *ऑरियस* का 14.5% और *विब्रियो स्पीप* के 13.7% ने एंटीबायोटिक दवाओं के लिए मल्टीड्रग प्रतिरोध (तीन या तीन से अधिक एंटीबायोटिक दवाओं के लिए प्रतिरोधी) प्रदर्शित किया। प्रतिरोधी पाए गए जीवाणु आइसोलेट को पीसीआर द्वारा एंटीमाइक्रोबियल रेजिस्टेंस जीन *अर्थात* ब्लैटम जीन की स्क्रीनिंग के लिए चुना गया था । 134 प्रतिरोधी आइसोलेट में से, ब्लैटम जीन को 102 आइसोले में परिलक्षित किया गया था, जिसने उच्च स्तर (76.1%) व्यापकता की। इसलिए, अध्ययन *एस्चेरिचिया कोलाई, स्टेफिलोकोकस ऑरियस,* और सुसंस्कृत चिंराट के विब्रियो एसपीपी के बारे में आवश्यक जानकारी प्रदान कर सकता है और आइसोले से जुड़े रोगाणुरोधी प्रतिरोध पर ज्ञान दे सकता है।

ABSTRACT

Antimicrobial resistance (AMR) of pathogens is a grave threat to global public health. The unregulated use of antibiotics in aquaculture is one of the causes of the emergence and spread of antimicrobial-resistant bacteria isolated from aquatic environments that pose a risk to aquatic organisms and human health. Shrimp aquaculture plays a significant role in AMR dissemination due to the extensive use of antibiotics as prophylactic and growth promoters. The current study was proposed to isolate and characterize the *Escherichia coli*, *Staphylococcus aureus* and *Vibrio spp* from the cultured shrimps of Maharashtra and Gujarat and to determine the phenotypic and genotypic traits of antimicrobial resistance associated with these selective bacteria. A total of 403 bacterial isolates were isolated and identified from 26 different farms. Out of 403 bacterial isolates comprising *Escherichia. coli* (113), *Staphylococcus. aureus* (62) and sucrose negative *Vibrio spp* (138) were isolated and characterized from 156 shrimp samples of different farm sources. Antimicrobial susceptibility pattern of the isolates against different antibiotics was carried out using the disk diffusion method. Among the three different bacteria isolated, a high prevalence of resistance was against the β-lactam class of antibiotics, and 134 isolates displayed resistance against Ampicillin along with Aztreonam and Cefotaxime. None of the isolates were found resistant to Trimethoprim/Sulfamethoxazole, Chloramphenicol, and Amoxicillin/Clavulanic acid. 28.5% of *E. coli* isolates, 14.5 % of *S. aureus*, and 13.7% of *Vibrio spp* displayed multidrug resistance (resistant to three or more than three antibiotic drugs) to the antibiotics tested. The bacterial isolates that were found resistant were selected for the screening of antimicrobial resistance gene *viz.,* blaTEM gene by PCR. Out of 134 resistant isolates, the blaTEM gene was amplified in 102 isolates, which indicated a high level (76.1%) of prevalence. Hence, the study could provide necessary information regarding *Escherichia coli*, *Staphylococcus aureus*, and Vibrio spp of cultured shrimps and could give knowledge on the antimicrobial resistance associated with the isolates.

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1. INTRODUCTION

Food and Agricultural Organization (FAO) defines aquaculture as the farming of aquatic organisms, including fish, molluscs, crustaceans, and aquatic plants. Aquaculture and fisheries sectors play a crucial role in meeting the future demand of animal protein, thus providing food security to more than 10% of the world population either directly or indirectly (Virdin *et al*., 2004; Bene *et al*., 2007; Allison, 2011). These sectors are the source of employment and income for many people in low-income families (Bene, 2006). Global fish production has reached almost 171 million tonnes in the year 2016. The first set value for fisheries and aquaculture production was nearly USD 362 billion, in which USD 232 billion was from aquaculture production. In the year 2016, world aquaculture production reached to 110.2 million tonnes (FAO, 2018). There is a decline in annual growth to 5.8 percent during the period of 2001-2006, but Africa has experienced double-digit growth from 2006-2010. Since 2000, global aquaculture no longer experiences the high annual growth rates as earlier of the 1980s and 1990s with 10.8 and 9.5 percent, respectively. The contribution of food fish from mariculture and coastal aquaculture in 2016 was 28.7 million tonnes (USD 67.4 billion), whereas inland aquaculture produced 51.4 million tonnes of food fish (FAO, 2018). Production of carps in freshwater and shrimps in brackish waters forms the leading part of aquaculture activities in significant areas.

In 2017, total crustacean production reported was 8.4 million tonnes, which are growing at an average annual rate of 9.92% per year since 2000, valued at US$61.06 billion (FAO, 2018). Shrimp is a highly traded commodity dominated by farmed tropical marine shrimp as a vital economic source for many developing countries. During the eighties, the growth of shrimp farming was slow and integrated shrimp culture with rice production that has evolved more considerable attention with many innovative notions (Wood *et al*., 1992; Wahab *et al*., 2003; Azad *et al*., 2009). Commercial rebounding of shrimp production began after the introduction of *Penaeus vannamei*. Among the Indian states, Andhra Pradesh contributes high shrimp production succeeded by West Bengal, Tamilnadu, Gujarat, and Odisha. Andhra

Pradesh, Gujarat, Tamilnadu, and Odisha prefer *Penaeus vannamei,* whereas *Penaeus monodon* is farmed in West Bengal and Kerala.

Intensified shrimp production is usually executed in a low geographical area with high stocking densities of hatchery-produced juveniles. There is an intense application of chemicals and antibiotic products in shrimp aquaculture. It was reported that many of the antibiotics used in hatcheries were clinically relevant (Holmstrom *et al*., 2003; Uddin & Kader, 2006; Thuy *et al*., 2011; Shamsuzzaman and Biswas, 2012; Ali *et al*., 2016; Chi *et al*., 2017; Hinchliffe *et al*., 2018).The shrimp industry was facing a severe problem with emerging bacterial diseases caused by *Vibrio species* and with necrotizing hepatopancreatic (NHP), which are major diseases hit in shrimp farms (Roque *et al*., 2001). *Vibrio species* are responsible for some of the diseases like bacterial erosion, Systemic vibriosis, Zoea II syndrome, and “white ball” in shrimps

“Antimicrobial” is the term referred to as the natural, (Friedman, 2015) semisynthetic, or synthetic substances (Ramos *et al.,* 2002; Fernandes *et al*., 2003) that act on various microorganisms like – bacteria (antibacterial), viruses (antiviral), fungi (antifungal) and protozoa (antiprotozoal). The typical use of antimicrobials in veterinary practice is more common than in human medicine for prophylactic treatment, which leads to the development of antibiotic-resistant bacteria (ARB) in the environment. Antibiotics used as growth promoters have been completely banned in Europe since 2006, this is still practiced in many countries, including the USA. In 2019, India banned the use of colistin in food-producing animals under the Drugs and Cosmetics Act, 1940 (Mahalmani *et al*., 2019).

Antibiotics have a vital role in modern medicine, acting as first-line treatment for bacterial infections (Davies *et al*., 2013). Antimicrobial resistance (AMR) emerged as one among the significant grave threats of public health due to the ineffectiveness of antimicrobials on the various microorganisms like bacteria, viruses, fungi, and parasites because of their ever-rising occurrence of infections. Antimicrobial resistance evolving from the usage of antimicrobials in aquaculture poses a risk to public health owing to either: Direct spread of resistance from the aquatic environment to humans by the infection of pathogenic bacteria that have acquired resistance (Eg. *Vibrio species* which are considered as pathogenic for

aquatic animals as well as humans). The indirect spread of resistance from the aquatic environment to humans is by horizontal gene transfer. The resistant bacteria serve as a reservoir of resistance genes that can be further disseminated and ultimately end up in human pathogens. For instance, there is the spread of resistance genes to *Escherichia coli* from aquatic pathogens *(Aeromonas species*) (Rhodes *et al*., 2000; Akinbowale *et al*., 2007). Augmentation of infections, enhanced frequency of treatment failure, and increased severity of infections are the major consequences for humans due to the transfer of antimicrobial resistance from aquaculture, which may affect the outcome with an escalated frequency of bloodstream, prolonged duration of illness, increased hospitalization or mortality.

E. coli belongs to family of Enterobacteriaceae that is widely distributed in the environmental samples, the intestine of humans and warm-blooded animals (Craven *et al.,* 2003; Abbott and Janda, 2006*. E. coli*, along with enteric bacteria like *Citrobacter, Enterobacter,* and *Klebsiella*, are termed as coliforms (Baylis *et al*., 2011), *E. coli* and Klebsiella comes under the group of faecal coliforms. Coliforms are known to occur naturally in the environment also. Hence, *E. coli* is considered as an indicator of environmental contamination as well as faecal contamination. Besides, *E. coli* is known to be pathogenic to humans and other mammals. *Staphylococcus aureus* is a Gram-positive coccus, ubiquitous in air, water, food, environment, and warm-blooded animals, including humans. *Staphylococci* are found in the nasal passage, respiratory passage, and on the hair and skin of humans and animals (ICMSF, 1996). *S. aureus* is considered as an indicator of environmental contamination of food (FDA, 2012). This organism is responsible for food poisoning in humans due to the ingestion of enterotoxins produced in food by some strains of *S. aureus* (Stewart, 2003). The occurrence of *S. aureus* organisms in food may indicate poor handling or sanitation. Antimicrobial resistance specially Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common problem, especially for human health. MRSA is the strain of *S. aureus* that has developed, multiple drug resistance to beta-lactam antibiotics including penicillin derivatives such as methicillin and oxacillin, and cephalosporins either through horizontal gene transfer or natural selection

Vibriosis is a major disease in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries. Based on phenotypic data, the major species causing vibriosis in shrimp are *V. alginolyticus, V. anguillarum, V. harveyi* and *V. parahaemolyticus*. Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) has caused large scale mortalities of farmed shrimp in China, Malaysia, Thailand and Vietnam and current understanding is that EMS/AHPNS has a bacterial etiology by a strain of *Vibrio parahaemolyticus* (Lightner *et al*., 2012; Zorriehzahra and Banaederakhshan, 2015). *V. parahaemolyticus*, like other vibrios, grows best under alkaline conditions, and so isolation from foods is facilitated by the use of media formulated with an alkaline pH (DePaola and Kaysner, 2004; Paydar *et al*., 2013).

WHO has recognized AMR as an urgent priority public health issue (WHO, 2018). It has attracted several stakeholders and the European Commission to frame Action Plan to tackle this issue (Davies and Gibbens, 2013; Smith *et al*., 2016). India has also framed its National Action Plan for the containment of AMR (GOI, 2017). WHO has classified several antimicrobial agents as critically important drugs for human medicine, which are also having potential utility in aquaculture during the meeting held in Canberra, Australia (WHO, 2005) and in Copenhagen, Denmark (WHO, 2007).WHO is recommending the farmers and food industry to impede the routine use of antibiotics for promoting growth and in the prevention of disease in healthy animals. The novel WHO recommendations aim to preserve the effectiveness of antibiotics vital in human medicine by slashing their excessive use in animals. To alter this AMR crisis, WHO through the coalition with FAO and OIE ('Tripartite Alliance') endorsed the 'One Health approach' and published the 'Global Action Plan (GAP) on antimicrobial resistance' in 2015 (WHO, 2015)

In India, a total of twenty-two antibiotics were banned for usage in aquaculture by Coastal Aquaculture Authority, including chloramphenicol, nitrofurans, sulphonamides, and glycopeptides, etc. Even though there has been an unregulated use of antibiotics as growth promoters and prophylactic drugs. The antibiotics that are most frequently used in aquaculture to combat bacterial diseases include oxytetracycline, florfenicol, sarafloxacin, and enrofloxacin. Globally, other antibiotics such as chlortetracycline, quinolones, ciprofloxacin, norfloxacin, oxolinic acid,

pefloxacin, sulfamethazine, gentamicin, and tiamulin are used. Various antimicrobials are also in use globally in addition to this. Chloramphenicol has now restricted for use in aquaculture in most countries, as a result of the danger of extreme human disease-related with the buildup of the residue due to chloramphenicol. This has brought about the confinement of its use in veterinary medicine to non-food use. Despite these restrictions, chloramphenicol has detected in national monitoring programs amid the past years, and these residues have caused safety concerns. Shrimp, prawns, and food products from aquatic animals were among the commodities in which the drug was detected (Ababouch *et al.*, 2005).

Antibiotic consumption in both humans and animals is increasing in low- and middle-income countries (Van Boeckel *et al.*, 2015; Klein *et al.*, 2018) and it is considered as greatest risk to health of humans and animals. Based on the present rate of spread of resistant infections, it was estimated that no of deaths may reach to 10 million people by 2050 with the economic loss of £60 trillion (O'Neill, 2014).

So, the present study was proposed with the following objectives:

- To screen and characterize selected bacterial isolates from *Penaeus vannamei*
- To study the phenotypic and genotypic traits associated with antimicrobial resistance of isolated selective bacteria.

REVIEW

OF

LITERATURE

2. REVIEW OF LITERATURE

2.1 Shrimp farming in India

Shrimp farming has risen in tropical and subtropical regions since the early 1980s, and Southeast Asia is the foremost leading region. Asian countries are contributing 75 % of world farmed shrimp production. The estimated estuarine area in India is 3.9 million hectares, in which (13%)1.2 million hectares are apt for shrimp farming covering ten states and union territories. Among them, the maximum area is located in West Bengal, followed by Gujarat and Andhra Pradesh. Andhra Pradesh contributes more than half of shrimp production in India, followed by Tamilnadu. Wild shrimp production was estimated at 450,000 MT whereas the cultured shrimp production was estimated at 426,500 MT for the FY 2014-2015 (USDA GAIN report, 2017)

2.1.1 Disease threats in shrimp farming

Despite the rapid diversification and intensification in global shrimp aquaculture, the farming industry came across with various pathogenic diseases in the past decades, which reported to cause devastating economic losses (Seibert *et al*., 2012). Globally, it is predicted that huge losses in shrimp aquaculture have been associated with viral pathogens which account for 60% mainly by White spot syndrome virus (WSSV), Infectious hypodermal and hematopoietic necrosis virus(IHHNV), Taura syndrome virus(TSV), Yellowhead virus (YHV), and Infectious myonecrosis virus(IMNV)(Flegel.,2012; Seibert *et al*., 2012). Bacterial pathogens have been attributed to 20% of disease losses, mainly due to Vibrio sp. (Ruangpan and Kitao.,1991; Moriarty.,1998). *Vibrio parahaemolyticus,* which is responsible for acute hepatopancreatic necrosis disease (AHPND), initially known as Early Mortality Syndrome, has led to devastating losses of more than 1 billion dollars in Asia with 100% mortality in post-larvae of shrimp. (FAO, 2013; Tran *et al*., 2013; Han *et al*., 2015; Thitamadee *et al*., 2016; Restrepo *et al*., 2018). Fungi and parasites have caused relatively low losses in contrast to viruses and bacteria.

2.1.2 *Penaeus vannamei*

Some marine shrimp species like whiteleg shrimp are included in inland aquaculture production due to their ability to grow in freshwater and saline waters (FAO, 2018). Globally the culture of penaeid shrimp is an economically vital activity in intertropical countries. The major problem for the decline in shrimp production is infectious diseases causing a disastrous collapse in the shrimp industry (Lightner, 1983; Vega-Villasante and Puente, 1993). *Penaeus vannamei* is native species of Pacific coast of Mexico and Central and South America (Wyban and Sweeny, 1991; Rosenberry, 2003), which has displaced the major shrimps especially *Penaeus monodon* due to its superior traits such as rapid growth rate, high survival rate, lower feed requirements and resistance to diseases. Whiteleg shrimp, *Penaeus vannamei* is one the major species of world aquaculture contributing to 53% of the total production of crustaceans (FAO, 2018)

2.2 Antimicrobials

According to the analysis of global antibiotic consumption in 2010, India stood forefront in total consumption of antibiotics with $12{\cdot}9 \times 10^9$ units ($10{\cdot}7$ units per person) (Van Boeckel *et al.*, 2014). In many areas, intensive and semi-intensive practices are followed to increase aquaculture production. These practices lead to the development of diseases, which led to an increase in the use of antimicrobials (Defoirdt *et al.,* 2007, 2011).

2.2.1 Mechanism of action of antimicrobials

The antimicrobial activity of most of the classes of antibiotics depends on the bacterial structure or metabolic process. Antibiotics can have selective effects on vital functions with minimal effects on the host. The effect of antimicrobial agents depends on their chemical nature and its structure

The mechanism of action of antimicrobial agents are as follows:

a. Inhibition of cell wall synthesis (e.g., beta-lactams and glycopeptide agents)

b. Interference with protein synthesis (macrolides and tetracyclines)

c. Inhibition of nucleic acid synthesis (fluoroquinolones and rifampin)

d. Disruption of bacterial membrane structure (polymyxins and daptomycin)

e. Blockage of the key metabolic pathway(trimethoprim-sulfamethoxazole)

a) Inhibition of cell wall synthesis

The peptidoglycan layer imparts structural strength and acts as a protective barrier for the bacterium (Sobhanifar *et al.,* 2013). Interference with bacterial cell wall synthesis occurs by the application of antibacterial drugs such as beta-lactams (penicillins, cephalosporins, carbapenems, and monobactams) and the glycopeptides (vancomycin and teicoplanin) (Neu,1992; McManus,1997). Beta-lactam agents work by interfering with the enzymes required for the synthesis of the peptidoglycan layer of the bacterial cell wall (Josephine *et al*., 2014) whereas vancomycin and teicoplanin works by binding to the terminal D-alanine residues of the growing peptidoglycan chain which averts the crosslinking steps needed for the stable cell wall synthesis (McManus,1997; Kahne *et al*., 2005)

b) Interference with protein synthesis

Antibiotics such as Macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins, and oxazolidinones target the bacterial ribosome, the site of cellular protein biosynthesis. Macrolides, aminoglycosides, and tetracyclines work by arresting the acquisition of aminoacyl-tRNA with the bacterial ribosome, so-called 30S ribosome inhibitors (Chopra *et al*., 1992; Schnappinger *et al*., 1996; Chopra and Roberts, 2001; Hong *et al*., 2014) whereas erythromycin, clindamycin, lincomycin, chloramphenicol, and linezolid, etc. are 50S ribosome inhibitors (Douthwaite, 1992; Katz and Ashley, 2005) acts by hindering either the initiation phase (Patel *et al*., 2001) or the elongation phase of protein translation(Menninger and Otto, 1982; Vannuffel and Cocito, 1996)

c) Inhibition of nucleic acid synthesis

Fluoroquinolones exert their activity by inhibiting the DNA synthesis, whereas rifampin by inhibiting the RNA synthesis. DNA topoisomerases are enzymes

that are essential for bacterial DNA synthesis. Topoisomerase IV is a potent decatenating enzyme that involves resolving interlinked daughter chromosomes and separates the daughter cells at the terminal stage of DNA replication. DNA gyrase involves in catalyzing the negative super-coiling of double-stranded closed-circular DNA (Zechiedrich and Cozzarelli,1995; Drlica and Zhao, 1997) Fluoroquinolones targets both the enzymes (DNA gyrase and Topoisomerase IV) thereby inhibits the DNA synthesis (Marians and Hiasa, 1997)

d) Disruption of bacterial membrane structure

Membrane-active antibiotics are specific in each microbial group based on lipid variation of the cell membrane. Daptomycin, a cyclic lipopeptide, acts on the cytoplasmic membrane and causes depolarization of calcium-dependent membrane potassium ion leakage. This results in cessation of macromolecular synthesis (synthesis of DNA, RNA, and proteins) and disruption of the cellular membrane in bacteria (Alborn *et al.*, 1991; Carpenter and Chambers, 2004). The polymyxins increase the bacterial membrane permeability and result in bacterial content leakage (Storm *et al.*, 1977) and also binds to the lipid moiety of the lipopolysaccharide in the bacterial cell disrupting bacterial cell membrane (Falagas *et al.*, 2010).

e) Blockage of the key metabolic pathway(trimethoprim-sulfamethoxazole)

Sulfonamides and trimethoprim (TMP) work by impeding the folic acid synthesis pathway, ultimately leading to the inhibition of DNA synthesis). Folic acid is essential in the metabolism of nucleic acid and amino acids. In this way, sulphonamides ultimately halt the production of nucleic acids (DNA and RNA) and amino acids (Talaro and chess, 2008).

2.2.2 Antibiotic usage in shrimp farming

Antibiotics used to combat bacterial diseases in shrimp farming includes oxytetracycline, sarafloxacin, enrofloxacin, florfenicol (Roque *et al.*, 2001; SotoRodríguez *et al.*, 2006) and also chlortetracycline, ciprofloxacin, oxolinic acid, norfloxacin, perfloxacin, sulfamethazine, quinolones, gentamicin, and tiamulin are used (Holmstrom *et al.*, 2003). Antibiotics are most frequently used as prophylaxis as

well as therapeutic drugs. Few farmers applied them as antiviral drugs to treat or avert viral diseases like white spot disease (Holmstrom *et al*., 2003). Prophylactic use of antibiotics in uninfected animals is more recurrent in hatcheries rather than in farms. (Smith, 2008, 2012; Zhang *et al*., 2011). The major reason for the huge occurrence of multiple antibiotic resistance (MAR) in hatcheries than in ponds is because of regular use of antibiotics as prophylactic agents for improving feed efficiency, enhancing growth rate, and for treating the bacterial infections (Vaseeharan *et al*., 2005) which results in antibiotic residues in culture environment that assist in occurrence and growth of resistant bacteria(Le *et al*., 2005). A study conducted on shrimp hatcheries of Bangladesh has revealed that 23 antimicrobial drugs were used at the rate of 80 kg per production cycle (Hinchliffe *et al*. 2018). There are restrictions in the application of antibiotics like chloramphenicol, furazolidone, fluoroquinolones, dimetridazole, nitrofurazone, and other nitrofurans which are not allowed to use at any stage of the production cycle (Defoirdt *et al*., 2007; Tittlemier *et al*., 2007)

2.3 Antibiotic resistance

Indiscriminate usage of antibiotics in fish farms, shrimp hatcheries, and ponds, either to treat the infections or as prophylactic purposes, may result in diffusion of antibiotics to the surrounding environment and aid in selective pressure for resistance development of bacteria in cultured fish and shrimps. For example, the Taiwan shrimp industry was collapsed due to the indiscriminate use of antibiotics, which resulted in the occurrence of resistant pathogens (Lin, 1989).

2.3.1 Antibiotic resistance of *E. coli*

Antimicrobial resistances for 80 *E. coli* strains were estimated with commercially available antibiotics. 56.25% strains(maximum) were resistant to ampicillin, and 2.5% strains(minimum) were resistant to chloramphenicol. The above 20% of isolated strains were resistant to 6 antibiotics (ampicillin, amoxicillin, cephalothin, ciprofloxacin, nalidixic acid, and tetracycline) (Kumaran *et al.,* 2010). Previous studies have reported that *E. coli* isolates from animals and from food products can harbor resistance determinants to many classes of antimicrobial agents, constituting an essential reservoir for transmissible resistance genes (Martel *et al*., 2008).

2.3.2 Antibiotic resistance in *Staphylococcus aureus*

Staphylococcus aureus is a perilous pathogen in humans due to their gradual rise in both community-acquired and hospital-acquired infections. Due to the incidence of multidrug-resistant strains, treatment of these infections has become more problematic. Despite the availability of effective antimicrobials as a therapeutic agent to Staphylococcus, the bacteria seem to rapidly acquire resistance to these drugs (Diekema *et al*., 2004). According to the study conducted on antimicrobial resistance prevalence in *Escherichia coli*, *Salmonella spp*. and *Staphylococcus aureus* that are isolated from fish samples in India showed that among the three, isolates of *S. aureus* exhibited highest (98%) multidrug-resistant(MDR) phenotypic pattern against three or more than three antimicrobial drugs of different combinations and classes. Fourteen antimicrobial agents were used for *S. aureus* susceptibility testing. (Saharan *et al.*, 2020). Shortly after the methicillin introduction in 1959 to overcome the obstacle of penicillin-resistant *Staphylococcus aureus* (PRSA), methicillin-resistant *S. aureus* (MRSA) strains were detected (Barber, 1961; Zhang *et al*., 2005). Staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element, harbors the methicillin resistance (*mecA*) gene, and other antibiotic resistance determinants have integrated into MRSA strains genome (Ito *et al*., 2001; Ma *et al*., 2002; Ito *et al*., 2004). These strains of MRSA have evolved as nosocomial pathogens (Crossley *et al*., 1979; Panlilio *et al*., 1992; Voss *et al*., 1994; Ayliffe *et al*., 1997) and source for community-acquired infections (Vandenesch *et al*., 2003; Lindsay *et al*., 2004)

2.3. Antibiotic resistance in *Vibrio species*

According to the study, *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are the species of *Vibrio* isolated from aquaculture water displayed high resistance rates ranging between 70 and 98.4% for carbenicillin, tetracycline, doxycycline, ampicillin, and mezlocillin while low to moderate resistance rate (32.8%) for kanamycin. High resistance rates were noticed for ampicillin, and among the three, V. cholerae expressed high resistance rates to 4 of 6 antimicrobials tested (Chikwendu *et al*., 2014). Prophylactic use of oxytetracycline in shrimp farms of Thailand has diminished the sensitivity of *Vibrio sp* (Nash *et al.,* 1992). A study

conducted on the evaluation of phenotypic resistance profile of *Vibrio sp* that are isolated from hatchery water and hepatopancreas of *L. vannamei* has revealed the occurrence of ampicillin resistance (45.2%) and resistant to tetracycline group (38.7%) (Rebouças *et al.*, 2011).

2.3.4 Mechanism of antibiotic resistance transfer

Bacterial resistome is the term used for a group of genes that encodes for resistant phenotypes, thus impedes the activity of antibiotics. It is mainly of two types, namely intrinsic and extrinsic resistome. Intrinsic resistome (innate) is acquired via mutations, and its occurrence is independent of preceding exposure to antibiotics (Martínez, 2012; Leclercq *et al.,* 2013; Galan *et al.,* 2013) whereas extrinsic resistome is acquired through horizontal gene transfer involving mobile genetic elements (Hu *et al.*, 2017; Bello-López *et al.*, 2019). Horizontal gene transfer is the phenomenon of resistance transfer through the exchange of genetic material between bacteria even if they belong to different genera, in which the one has never been exposed to the antibiotic (Shoemaker *et al.*, 2001; Li et al., 2018). The genetic material is received in the form of plasmid or transposon, which are mobile genetic elements that are easily transferable between bacteria. There are three major mechanisms of horizontal gene transfer – conjugation, transformation, and transduction. Conjugation is considered as the most feasible mechanism for antibiotic-resistant genes (ARGs) dissemination (Von Wintersdorff *et al.*, 2016)

2.4 Molecular characterization of antimicrobial resistance

Bacteria's genome determines its resistance like other phenotypic characteristics, which may change as the result of mutation or by the acquisition of new genetic material. Resistance to many antibiotics does not arise by mutation of the bacterial chromosome, and it can acquire new genes whose products affect resistance by various mechanisms. The observance of co-selection that the use of one antibiotic can lead to increased resistance to itself and another, an unrelated antibiotic. The animal gastrointestinal tract serves not only as of the main reservoir and essential site of bacterial multiplication but also as a place for the exchange of genetic information (Wegener *et al.*, 1999). The spread of antibiotic resistance occurs in two ways, i.e., as a result of infectious drug resistance genes spreading and, to a

large extent, resistant bacteria spreading. Resistant bacteria can transfer the resistance to other bacteria (even to bacteria of different genera) that have never been exposed to the antibiotic. This event is known as horizontal gene transfer (Shoemaker *et al*., 2001). Penicillin and cephalosporins (ß-lactam antibiotics) are mostly used, so resistance to these antibiotics is common; after discovering ß-lactamase inhibitors, researchers thought that the resistance problem was solved. Unfortunately, bacteria have evolved new mechanisms of resistance to overcome the effects of ß-lactamase inhibitors (Therrien and Levesque, 2000). 16s rRNA, Random Amplification of Polymorphic DNA (RAPD) are molecular techniques used for isolated antibiotic-resistant organisms' identification, which are essential for effective antimicrobial and supportive therapy (Sahilah *et al*., 2014). Using the non-specific culture methods and culture-independent real-time PCR, the abundance of the antimicrobial resistance has revealed, and a broad spectrum of non-pathogenic beneficial bacteria was found carrying transmissible AMR genes (Duran and Marshall, 2005; Wang *et al.,* 2006; Manuzon *et al.,* 2007)

2.5 Bacterial microflora of shrimp

2.5.1 *Escherichia coli*

Escherichia coli belongs to the Enterobacteriaceae family, and it is naturally distributed within the environmental samples, in all warm-blooded animals intestinal tracts, and can be easily spread in different ecosystems through the food chain and water. *E. coli*, along with enteric bacteria like *Citrobacter, Enterobacter*, and *Klebsiella,* are termed coliforms with the capability of lactose *(*Costa *et al*., 2009). *Escherichia coli* can exchange genetic material with other bacterial species, and these bacteria may transfer antibiotic resistance genes to transient bacterial pathogens causing diseases in humans (Alexander *et al.,* 2010). *Escherichia coli* serotype 0157: H7 has recently emerged as a significant foodborne pathogen causing haemorrhagic colitis in humans in Canada and the United States (Johnson *et al*., 2000, Pudden *et al.,* 1985, Riley, 1983).

2.5.2 *Staphylococcus aureus*

*Staphylococcus aureus i*s a Gram-positive, catalase-positive, facultative anaerobes that occur in clusters. The cell wall consists of peptidoglycan (linked polysaccharide subunits), and other structural components include polysaccharide capsules, and surface proteins (for example, Protein A) (Lowy, 1998) *Staphylococci* can cause food poisoning and shock by the release of enterotoxins (Marrack and Kappler, 1990). Staphylococci are commensal on the skin and mucous membranes of humans and animals. During 1982 and 1983, *Staphylococcus aureus* caused eye disease in silver carp, *Hypophthalmichthys molitrix* which resulted in massive mortalities (Shah and Tyagy, 1986)

Staphylococcus aureus was associated with eye disease during 1982 and 1983, causing mortalities among silver carp, *Hypophthalmichthys molitrix*, at a farm in India (Shah and Tyagy, 1986). Two species, *S. aureus* and *S. epidermidis* have been reported as causing staphylococcosis in fish. The description of the disease was not exhaustive, but typical signs included exophthalmia, congestion, and ulceration on the tail (Kusuda and Sugiyama, 1981). *S. aureus* raises the concern as a pathogen because of its natural virulence and the ability to adjust to different environmental conditions (Lowy, 1998).

2.5.3 *Vibrio species*

Vibrios are short Gram-negative rods, which are often curved and actively motile by a single polar flagellum. Bacteria of the genus Vibrio are commonly found in coastal and estuarine waters with high salinity and temperatures ranging between 10˚C to 30˚C (Strohl *et al.*, 2004; Murray *et al.*, 2004; Manjusha *et al.*, 2005). Some Vibrio strains are pathogenic and can cause Vibriosis, a severe infectious disease in both wild and cultured fish and shellfish. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, and *V. splendidus*, are known vibrio spp that are associated with diseased shrimp (Jayasree *et al.*, 2006, Chikwendu *et al.*, 2014).

2.6 Hazard characterization

2.6.1 Human health risks

Usage of anti-microbial agents in food animals is correlated with the anti-microbial resistance in bacteria isolated from humans like *Salmonella, Campylobacter, Escherichia coli,* and other bacteria (Angulo *et al.*, 2004). Enormous amounts of antibiotics among farm animals and in aquaculture have been used as growth promoters as well as prophylactic and therapeutic drugs, augmenting the selective pressure on both commensal and pathogenic microorganisms that infect humans through direct contact or the food chain and indirectly by farm effluents and transmit their resistance genes to human flora or allied human pathogens. (Heuer *et al.*, 2009; Liebana *et al.*, 2013) Accumulation of antimicrobial resistance amidst the bacteria will escort human health consequences like high severity of infections, increased hospitalization, and mortality rates. There is an exchange of antibiotic resistance genes amongst the bacteria of the aquatic environment and terrestrial environment, which includes human and animal bacterial pathogens (Sørum, 1998; Rhodes *et al.*, 2000; Schmidt *et al.*, 2001; Sørum, 2006). Consumption of edible tissues of shrimp with accumulated antibiotic residues may alter human gut microflora and lead to allergic reactions and food poisoning (Ma *et al.*, 2006).

Vibrio cholera, the causative agent of epidemic cholera of Ecuador, which started in 1992, seemed to be acquired antibiotic resistance by interacting with antibiotic resistance bacteria selected through the overuse of antibiotics in Ecuadorian shrimp industry (Weber *et al.*, 1994). Vibrio sp are serious pathogens in shrimp farming, which are often associated with humans. For example, *Vibrio vulnificus, which is responsible for seafood associated deaths in the US (*Feldhusen *et al.,* 2000; Doyle *et al.*, 2019), and *Vibrio parahaemolyticus,* the causative agent of food-borne gastroenteritis (Nair *et al.*, .2007; Li *et al.*, 2014). Handling antibiotics with bare hands during preparation of medicated feeds for shrimps leads to serious health issues such as aplastic anaemia due to chloramphenicol exposure, whereas skin dermatitis from exposure of sulfonamide. (Brown, 1989; Gräslund *et al.,* 2003).

2.6.2 Environmental risks

Many of the antibiotics having bio accumulative and toxic properties are employed in shrimp farming may culminate in water or sediment; therefore, these are considered as persistent and slightly to highly acutely toxic. Incidence of vast amounts of antibiotics in sediments and water of aquatic environment will affect the flora and plankton, which in turn alter the microbiota diversity(Davies *et al* ., 1999; Miranda and Zemelman, 2001; Cabello, 2003; Hunter-Cevera *et al* ., 2005; Sørum, 2006). These alterations can be enhanced by the eutrophication due to augmented input of N, C and P yielded by the unconsumed food and fish excreta in the aquaculture environment (Goldburg *et al*., 2001; Hunter-Cevera *et al*., 2005). The environment has numerous routes of entry of huge amounts of antibiotic residues and resistant bacteria as a consequence of anthropogenic activities such as drug manufacturing, sewage from the community or hospitals, agricultural effluents and wastewater treatment(Heuer *et al*., 2009; Finley *et al*., 2013) which accelerates the chance of recruitment of resistance genes into clinically relevant pathogens(Knapp *et al*., 2011), converts the environment into a vast reservoir for antibiotic resistance genes and thereby increasing environmental pollution(Roca *et al.,* 2015). Degradation studies conducted on antibiotics that are known to be persistent in marine aquatic sediments showed results varying with temperature, light intensity, depth in sediment, etc. (Samuelsen, 1989; Gavalchin and Katz, 1994; Samuelsen *et al*., 1994; Weston, 2000). Sulfadimethoxine, ormetoprim, and chloramphenicol seem to be short-lived in marine sediments (Lai *et al*., 1995; Capone *et al.,* 1996). Studies conducted on bioaccumulation of antibiotics in fish and sediments have been revealed that residues of oxytetracycline and oxolinic acid were found in wild fauna (Björklund et al., 1990; Samuelsen et al., 1992; Capone et al., 1996). Antibiotics such as ciprofloxacin, oxolinic acid, tetracycline, oxytetracycline, trimethoprim, mecillinam, etc.has shown toxic effects on algae and aquatic invertebrates (Lützhøft *et al*., 1999; Halling-Sørensen,2000; Halling-Sørensen *et al*., 2000; Wollenberger *et al.,* 2000) and overflow of antibiotics in shrimp culture may show the effect on nearby aquatic ecosystems.

MATERIAL

&

METHODS

3. MATERIAL AND METHODS

3.1 Site of experiment

The present study was performed in the Aquatic Environment and Health Management Division, ICAR-Central Institute of Fisheries Education, Mumbai, India.

3.2 Media and Ingredients

The list of equipment, chemicals, and consumables used in this study are given in Appendix I. Culture media used in the present study were purchased from Hi-Media (India). The composition of media and reagents used for the study are given in Appendix II. Culture media used for the study were sterilized by autoclaving at 15lb pressure at 121℃C for 15min.

3.3 Preparation of Glasswares and plastic petri plates

All the glasswares and plastic petri plates were soaked in mild detergent solution for 5-10minutes followed by washing thoroughly in tap water and rinsing with distilled water. After air drying, glass wares were sterilized in a hot air oven at 180℃ for two hours and plastic petri plates were autoclaved at 121℃, 15lbsfor 15 minutes. Microcentrifuge tubes, micro tips PCR tubes etc. were sterilized by autoclaving at 121℃, 15 lbs for 15 minutes.

3.4 Sample collection

A total of 156 live shrimps were collected from shrimp farms located in Thane and Surat districts of Maharashtra and Gujarat respectively. From Thane, a total of 14 farms and in Surat, 12 farms were covered. The details of sampling sites are given in table 1. The details of the farms and samples collected are given in Appendix III.

Table 1: Sampling sites at Thane and Surat districts

S.No	Sampling stations	Latitude	Longitude
1	Dahanu, Dhumket	19°56'52.0" N	072°43'25.4" E
2	Dahanu, Dhumket	19°57'12.660"N	072°43'26.880" E
3	Dahanu, Chandigaon	19°55'46.560"N	072°43'46.560" E
4	Dahanu, Agwan	19°56'36.960"N	072°46'28.260" E
5	Palghar, Saphale	19°31'40.980" N	72°48'20.400" E
6	Palghar, Saphale	19°32'15.5" N	72°45'30.0" E
7	Dahanu,Bade Pokharan	19°57'34.560"N	72°42'0.5400" E
8	Dahanu, Sawata	19°57'34.6" N	72°41'59.5" E
9	Dahanu, Bordi	20°06'52.740"N	72°44'24.000" E
10	Vasai, Chandrapada (CT)	19°22'38.9" N	072°51'16.0" E
11	Palghar, Mithagar	19°32'11.100"N	072°47'19.200" E
12	Palghar, Jalsar	19°31'48.4" N	72°48'22.6" E
13	Palghar, Kharekuran	19°33'10.560"N	72°47'8.4600" E
14	Vasai, Malaji Pada	19°21'33.7" N	072°49'18.6" E
15	Olpad, Kundiyana	21°19'9.9000"N	72°39'33.780" E
16	Olpad, Kundiyana	21°19'53.280"N	72°39'34.560" E
17	Olpad, Saras	21°19'41.880"N	72°39'30.960" E
18	Olpad, Kapasi	21°20'27.840"N	72°42'56.880" E
19	Olpad, Kapasi	21°20'26.280"N	72°40'4.0200" E
20	Olpad, Mor	21°23'6.2400"N	72°39'49.920" E
21	Olpad, Mor	21°23'12.360"N	72°39'43.140" E

22	Olpad, Mor	21°21'52.680"N	72°40'7.0200" E
23	Olpad, Mor	21°23'17.640"N	72°40'2.8800" E
24	Olpad, Delasa	21°21'35.0" N	72°40'48.7" E
25	Olpad, Sarsana	21°04'41.7" N	072°47'20.3" E
26	Olpad, Sarsana	21°04'55.3" N	72°47'40.1" E

3.5 Sample processing

Live shrimp samples were transported to the laboratory in Amies transport media maintained <10°C. They were dissected aseptically using sterile scissors and forceps. Whole shrimps were processed for isolation of *Escherichia coli, Staphylococcus aureus*, and *Vibrio spp.*

3.6 Isolation of target bacteria

3.6.1. *Escherichia coli*

3.6.1.2 Sample preparation and enrichment

Shrimp samples were homogenized in MacConkey broth using pestle and mortar. From each sample, a volume of 1 ml was transferred to EC broth containing Durham tube and incubated at 44.5°C for 24±2 hours for enrichment. Post incubation, the presence of gas bubble in inverted Durham 's tube was considered as positive for the growth of *E. coli.*

3.6.1.3 Isolation on L-EMB Agar media

Overnight cultures from EC broth were taken and streaked onto L-EMB agar plate; incubated at 35 °C for 20 hours. Post incubation, colonies were picked based on the morphological characteristics of target species for further characterization..

3.6.2 *Staphylococcus aureus*

Isolation and identification of *Staphylococcus* aureus was performed by following the SOP for Network Programme on Antimicrobial Resistance (AMR) in Fisheries (ICAR-NBFGR 2018).

3.6.2.1 Sample preparation and enrichment

The collected samples were homogenized in tryptone soya broth containing 10% NaCl and 1% sodium pyruvate and incubated at 37^0 C for 24±2 h in shaking incubator for enrichment.

3.6.2.2 Isolation on Baird Parker agar media

Post incubation, loop full of culture was taken and streaked onto Baird parker (BP) agar plate containing egg yolk and potassium tellurite. Based on the colony characteristics, plates were incubated at 37°C for 48 h, and colonies were picked for further studies

3.6.3 *Vibrio* spp.

3.6.3.1 Sample preparation

Whole shrimp samples were homogenised in Phosphate Buffered Saline (PBS) and serially diluted in autoclaved 0.85% saline water.

3.6.3.2 Isolation on TCBS agar

A volume of 0.1 mL from each dilution was inoculated on thiosulfate citrate bile salt sucrose (TCBS) agar plates. The samples were spread over the surface with sterile bent glass rods. The plates were left undisturbed for 30 minutes and incubated at 35±2°C for 18-24 hours.

3.6.3.3 Identification of *Vibrio spp.*

GREEN colonies (round, opaque) measuring approx. 2-3 mm diameter from the TCBS agar plate were selected. The selected colonies were streaked on TSA-2% NaCl agar plates for isolating pure colonies of *Vibrio* and incubated overnight at 35 ±2°C. The pure cultures were used for further studies.

3.7. Biochemical characterization of target bacteria

Biochemical characterization of *S. aureus*, *E. coli* and *Vibrio* spp. were performed following the SOP for Network Programme on Antimicrobial Resistance in Fisheries and Aquaculture by ICAR-NBFGR 2018.

3.7.1. Catalase and Oxidase test

Bacterial colonies picked from the selective media as discussed in section 3.6.1, 3.6.2 and 3.6.3 using sterile loops and streaked on glass slides containing 3% hydrogen peroxide solution. The isolates producing bubbles within 15 seconds of inoculation were identified as positive for the presence of catalase. Presence of the intracellular oxidase enzyme in the isolates were performed using filter papers pre-moistened with Kovac 's cytochrome oxidase reagent. For this, a sterile platinum loopful of isolated bacterial cultures were smeared on the test paper. Development of dark purple or blue colour within a few seconds indicated a positive test and oxidase-negative isolates were indicated by lack of colour change.

3.7.2. Biochemical characterization of *Escherichia coli*

The colonies picked from L-EMB agar plates were further subjected to IMViC (Indole, Mannitol, Voges Proskauer and Citrate Utilization) test.

3.7.2.1 Indole test

Bacterial colonies were picked using sterile loops and inoculated into sterile indole broth and incubated at 30°C for 18-20h. A drop of Kovacs' reagent was added to the media after incubation. Isolates which are positive for indole production were indicated by a production of a layer of pink colour, and negative isolates were indicated by yellow colour.

3.7.2.2 Methyl red (MR) and Voges-Proskauer (VP) tests

Single colonies of the isolates were inoculated into MRVP broth, and the test tubes were then incubated at 37°C for 48h. After 48h, 1ml of the broth was aliquoted into another sterile test tube for the MR test, and the main one was used for the VP test.

For MR test, five drops of Methyl red indicator were added into the broth containing culture and the results were observed immediately. MR positive indicated the production of red colour, and negative isolates indicated the production of a yellow colour.

For the VP test, two reagents solution A and solution B of 0.6ml and 0.2ml respectively were added. A small crystal of creatinine was added and allowed to stand up to 4h. A pink iodine colour indicates a positive VP test.

3.7.2.3 Citrate test

A little of the culture was streaked on to the Simmons's citrate agar slants and incubated at 37°C for 48h, and then the results were read after the incubation. The colour change from green to blue is indicated as positive.

3.7.3 Biochemical characterization of *Staphylococcus sp.*

3.7.3.1 Coagulase test

For performing the coagulase test, a volume of 0.5 ml of the reconstituted coagulase plasma with ethylenediaminetetraacetate (EDTA) (0.15%) was taken into small test tubes. Two drops of the overnight suspected *S. aureus* cultures were added and mixed thoroughly. The test tubes were incubated at 35°C and examined periodically over a 6hr period for clot formation. Only firm and a complete clot that stays in place when the tube is tilted or inverted were considered positive.

3.7.3.2 Anaerobic utilization of mannitol

Loopful of the overnight culture of suspected *S. aureus* were streaked on to the mannitol agar plates and incubated for five days at 37°C.The production of yellow pigment indicated a positive reaction.

3.7.4 Biochemical characterization of *Vibrio* spp.

3.7.4.1 Nitrate reduction test

The microorganism's' ability to reduce nitrate to nitrite was tested using nitrate reduction test kit. For this, a drop of culture grown in nitrate broth (37°C, 24h) was taken on a petri plate. A disc --Part A-0.5% α- Naphthylamine in dil. HCl) was placed on the culture. Further the solution -Part B-0.8% Sulphanilic Acid in dil. HCl was added. A development of red colour was considered positive for nitrate reduction. In absence of red colour, the organism was considered as negative for nitrate reduction.

3.7.4.2. Hugh and Leifson's oxidative / fermentative metabolism

The tubes containing H&L O/F medium were stab inoculated and incubated at 37°C for 24h. The reaction was noted as fermentative when the entire media turned yellow. The oxidative reaction was characterized by yellow colour only in the upper portion of the media. Simultaneously, another set of H&L O/F medium tube was stab inoculated, overlaid with sterile liquid paraffin, and incubated at 37°C for 24h. The appearance of yellow colour in these tubes was taken as positive for fermentation.

3.7.5 Gram's staining

Staining of isolated bacteria were performed following the protocol of Christian Gram, 1884. Briefly, young cultures were emulsified with sterile water on glass slides and spread uniformly. The slides were air dried and heat fixed. The smears were flooded with Gram's crystal violet for 1 min followed by washing with tap water. Post washing, the slides were flooded with Gram's iodine for 1 min and were again washed with tap water. Destaining was done by adding alcohol dropwise until washings were free of violet colour and were washed with tap water. Counterstaining of the slides were done with Safranine for 1 min and were air dried before visualization under the microscope. The slides were observed under the microscope using an oil immersion objective (100X). Cells stained violet, bluish violet or bluish purple were identified as Gram +ve and those stained red as Gram -ve.

3.8 Preservation of bacterial isolates

Even though a bacterial colony is assumed to have arisen from a single cell in the inoculums, occasionally mixed growth is possible. Hence, the cultures were purified by picking pure colonies using sterile loop and streaked on to the surface of sterile nutrient agar slants. The slants were arranged in an incubator at 30°C for 12-16h. The slants were then wrapped in parafilm and stored at 4°C for further analysis.

3.9 Confirmation of bacterial isolates by using the VITEK 2 Compact system

3.9.1 Reagent Cards

The reagent cards consist of 64 wells, each provided with an individual test substrate. Substrates measure different metabolic activities such as acidification, alkalinisation, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that inhibits contact with the organism-substrate admixtures. Each card consists of a pre-inserted transfer tube used for inoculation. Cards with bar codes contain information on product type, lot number, expiration date, and a unique identifier that can be connected to the sample either before or after loading the card onto the system. The two different types of cards are used like the gram-negative and gram-positive cards, and the 64 tests of each card are described in Appendix IV.

3.9.2 Suspension Preparation

With sterile swab, a sufficient number of colonies of a pure culture were taken into clear plastic (polystyrene) test tube with 3.0 ml of sterile saline solution (pH 4.5 to 7.0,0.45% to 0.50% NaCl)

3.9.3 Inoculation

Identification cards are inoculated with bacterial suspensions using an integrated vacuum apparatus and placed into a rack (cassette). The identification card is positioned in the neighbouring slot while inserting the transfer tube into the suspension tube. The cassette can accommodate up to 10 test tubes (VITEK 2 Compact). The filled cassette is placed manually (VITEK 2 compact) into a vacuum chamber station. After the vacuum is applied and the air is re-introduced into the

station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells.

3.9.4 Card Sealing and Incubation

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card before loading into the carousel incubator. All card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator, transported to the optical system for reaction Data is collected after the entire incubation period.

3.10 Antimicrobial Susceptibility Test

Isolated bacterial colonies were subjected to Antimicrobial susceptibility testing by disk diffusion method as proposed by Kirby &Baeur (1966). The antibiotics utilized in the present study are listed in table 2.

3.10.1 Preparation of bacterial inoculum

Bacterial isolates were inoculated in 5ml sterile nutrient broth tubes and incubated overnight at 30^0 C. Turbidity of the cultures were compared with 0.5 MacFarland standard and adjusted by adding sterile normal saline or more bacteria.

3.10.2 Inoculation on Mueller-Hinton (MH) plate

Sterile cotton swabs were dipped into the culture. The excess was removed from the swab by gently pressing it against the side of the tube. The suspension was then streaked onto the surface of the Mueller-Hinton agar plates. Streaking was done in all the three planes to make a bacterial mat of equal thickness on the surface of the agar.

3.10.3 Antibiogram

The antibiotics to be tested were segregated in aseptic conditions. Using sterile forceps, the antibiotic disks were placed on the surface of the agar and pressed gently. The plates were then kept for incubation within 30mins at 30℃ for 24h. After incubation, the results were determined by measuring the diameter of zone

of inhibition, which included the diameter of the antibiotic disk with the Antibiotic Zone scale. The measurements were analysed using WHONET 5.6 software.

Antibiotics used in the study

Table 2: Antibiotics used for *Escherichia coli*

Code	Antibiotic name	Antibiotic class	Antibiotic subclass	Breakpoints
AMP	Ampicillin 10 µg	Penicillins	Aminopenicillin	14 - 16
AMC	Amoxicillin/Clavulanic acid 20/10 µg	Beta-lactam+Inhibitors	-	S >= 18
CAZ	Ceftazidime 30 µg	Cephems	Cephalosporin III	18 - 20
CRO	Ceftriaxone 30 µg	Cephems	Cephalosporin III	20 - 22
CTX	Cefotaxime 30 µg	Cephems	Cephalosporin III	23 - 25
FEP	Cefepime 30 µg	Cephems	Cephalosporin IV	19 - 24
FOX	Cefoxitin 30 µg	Cephems	Cephamycin	15 - 17
CPD	Cefpodoxime 10 µg	Cephems-Oral	Cephalosporin	18 - 20
ATM	Aztreonam 30 µg	Monobactams	-	18 - 20
IPM	Imipenem 10 µg	Penems	Carbapenems	20 - 22
AMK	Amikacin 30 µg	Aminoglycosides	-	15 - 16
GEN	Gentamicin 10µg	Aminoglycosides	-	13 - 15
NAL	Nalidixic acid 30 µg	Quinolones	Quinolone	14 - 18
CIP	Ciprofloxacin 5 µg	Quinolones	Fluoroquinolone	22 - 25
SXT	Trimethoprim/Sulfamethoxazole 1.25/23.75 µg	Folate pathway inhibitors	-	11 - 15-

COL	Colistin 10 µg	Lipopeptides	-	S >= 11
ERY	Erythromycin 15 µg	Macrolides	14-Membered ring	14 - 22
CHL	Chloramphenicol 30 µg	Phenicols	-	13 - 17
TCY	Tetracycline 30 µg	Tetracyclines	-	12 - 14

Table 3: Antibiotics used for *Staphylococcus aureus*

Code	**Antibiotic name**	**Antibiotic class**	**Antibiotic subclass**	**Breakpoints**
PEN	Penicillin G 10 µg	Penicillins	Penicillin	S >= 29
AMP	Ampicillin 10µg	Penicillins	Aminopenicillin	S >= 29
AMC	Amoxicillin/Clavulanic acid 20/10 µg	Beta-lactam+Inhibitors	-	S >= 20
FEP	Cefepime 30 µg	Cephems	Cephalosporin IV	15 - 17
FOX	Cefoxitin 30 µg	Cephems	Cephamycin	S >= 22
ATM	Aztreonam 30 µg	Monobactams	-	18 - 20
IPM	Imipenem 10 µg	Penems	Carbapenems	14 - 15
AMK	Amikacin 30 µg	Aminoglycosides	-	15 - 16
GEN	Gentamicin 10 µg	Aminoglycosides	-	13 - 14
NAL	Nalidixic acid 30µg	Quinolones	Quinolone	14 - 18
CIP	Ciprofloxacin 5 µg	Quinolones	Fluoroquinolone	16 - 20
SXT	Trimethoprim/Sulfamethoxazole 1.25/23.75 µg	Folate pathway inhibitors	-	11 - 15

ERY	Erythromycin 15 µg	Macrolides	14-Membered ring	14 - 22
LNZ	Linezolid 30µg	Oxazolidinones	-	S >= 21
CHL	Chloramphenicol 30µg	Phenicols	-	13 - 17
TCY	Tetracycline 30µg	Tetracyclines	-	18 - 22

Table 4: Antibiotics used for *Vibrio spp.*

Code	**Antibiotic name**	**Antibiotic class**	**Antibiotic subclass**	**Breakpoints**
AMP	Ampicillin 10µg	Penicillins	Aminopenicillin	14 - 16
CAZ	Ceftazidime 30µg	Cephems	Cephalosporin III	18 - 20
CTX	Cefotaxime 30µg	Cephems	Cephalosporin III	23 - 25
FEP	Cefepime 30µg	Cephems	Cephalosporin IV	19 - 24
FOX	Cefoxitin 30µg	Cephems	Cephamycin	15 - 17
ATM	Aztreonam 30 µg	Monobactams	-	18 - 20
IPM	Imipenem 10µg	Penems	Carbapenems	20 - 22
MEM	Meropenem 10µg	Penems	Carbapenems	20 - 22
AMK	Amikacin 30 µg	Aminoglycosides	-	15 - 16
GEN	Gentamicin 10µg	Aminoglycosides	-	13 - 14
NAL	Nalidixic acid 30 µg	Quinolones	Quinolone	14 - 18
CIP	Ciprofloxacin 5 µg	Quinolones	Fluoroquinolone	22 - 25

SXT	Trimethoprim/Sulfamethoxazole 1.25/ 23.75 µg	Folate pathway inhibitors	-	11 - 15
CHL	Chloramphenicol 30 µg	Phenicols	-	13 - 17
TCY	Tetracycline 30µg	Tetracyclines	-	12 - 14

3.11 Molecular confirmation of *Vibrio* spp.

3.11.1 Template preparation

Pure culture of *Vibrio* spp. on nutrient agar slants were used to extract crude DNA following the boiling cell extraction method (Ahmed *et al.*, 2005). The bacterial culture was suspended in 500µl of 1X TE Buffer and vortexed shortly. The suspension was heated at 95^0 C for 10min and then was chilled at -20°C for 10min. The cell debris was pelleted by centrifugation at 13400 g for 10min and 5µl of clear supernatant was used as the DNA template in a PCR. The details of primers used is given in table no.5

3.11.2 Polymerase Chain Reaction (PCR)

PCR was performed for amplifying the 16SrRNA *Vibrio* genus specific primers in a thermocycler (Applied Biosystems, USA). Reaction volume of 25µl comprised of 12.5 µl of PCR master mix (2X), 0.6µl each of forward and reverse primers, 8.3µl of De ionized water and 3µl of template DNA. The reagents were thoroughly mixed and subjected to the following conditions. Initial denaturation at 95^0 C for 4 min, 25 cycles of denaturation at 94^0C for 1 min, annealing at 53^0C for 1 min and extension at 72 ^{0}C for 1 min and final extension at 72^0C for 10 min.

3.12 Molecular methods for characterization of AMR

3.12.1 Template preparation

Bacterial culture on nutrient agar slants was used to extract crude DNA by the use of the boiling cell extraction method. A single colony of bacterial cells was suspended in 500µl of 1X TE buffer and vortexed shortly. The suspension was heated at 950 C for 10min and then was chilled in ice for 10min. The cell debris was pelleted

by centrifugation at 13400 g for 10 min, and clear supernatant of 5µl DNA template was used as template for the PCR.

3.12.2 PCR targeting AMR genes

The PCR was performed using specific primers described in the table below for the blaTEM gene. The reaction volume of 25µl comprised of 12.5 µl of PCR master mix (2X), 0.6µl each of forward and reverse primers, 8.3µl of De ionized water and 3µl of template DNA. The reagents were thoroughly mixed and subjected to the following conditions. The reaction was carried out using the following cycling conditions. Initial denaturation at 94^{0}C for 10 min, 30 cycles of denaturation at 94^{0}C for 40 s, annealing at 60^{0}C for 40 sec and extension at 72 ^{0}C for 1 min and final extension at 72^{0}C for 7 min.

3.13 Visualization of PCR products

The PCR products were mixed with tracking dye, loaded on 1.5% agarose gels containing ethidium bromide (0.5µg/ml) along with suitable DNA ladders, electrophoresed at 70V. The gels were visualized under UV light using a gel documentation system (Syngene, UK) post electrophoresis.

Table 5: Primers used in the study

Target organism/ gene	**Sequence (5' to 3')**	**Amplicon size**	**Reference**
Vibrio genus 16S rRNA	5'-CGG TGA AAT GCG TAG AGA T– 3'	663 bp	Tarr *et al.*, 2007
	5'-TTA CTA GCG ATT CCG AGT TC– 3'		
blaTEM	CATTTCCGTGTCGCCCTTATTC	800bp	Dallenne *et al.*, 2010
	CGTTCATCCATAGTTGCCTGAC		

RESULTS

4. RESULTS

Escherichia coli, Staphylococcus aureus and *Vibrio species* (sucrose negative) were isolated from cultured shrimps of Maharashtra and Gujarat farms. The isolates were identified using microbiological techniques. Antimicrobial susceptibility pattern of the isolates was performed using disk diffusion method. The bacterial isolates, which showed resistance to the antibiotics, were screened for the presence of antimicrobial resistance genes.

4.1 Isolation of bacteria

A total of 403 bacterial isolates were isolated and identified from 26 different farms. From all positive samples, bacterial isolates were picked for further studies. These isolates were clinically characterized by various systematic biochemical tests, by VITEK 2 compact system (*E. coli* and *Staphylococcus aureus*) and molecular confirmation (*Vibrio spp.*).

4.1.1 Colony morphology

The size of the colony of all the isolates was ranging from 1-3 mm.

Table 6: Colony morphology of bacterial isolates

S. No	Isolate name	Colony morphology
1.	*Escherichia coli*	Dark centered and flat, with green metallic sheen
2.	*Staphylococcus aureus*	Black shiny colonies with light coloured (off white) margin surrounded by an opaque zone.
3.	*Vibrio species* (non-sucrose fermentative)	Green/Bluish-green centered, round, opaque colonies.

4.1.2 Presumptive identification of bacterial isolates

Among 403 bacterial isolates, 276 were found gram negative and 127 isolates were gram positive.

4.1.3 Biochemical characteristics

The results of the biochemical reactions of the isolates are given in the tables

Table 10: Biochemical reactions results of *E. coli* isolates

Characteristics	**Reaction**
Gram staining	$Gram^{-ve}$ rods
Catalase	Positive
Oxidase	Negative
Indole production	Positive
Methyl red test	Positive
Voges-Proskauer (VP) test	Negative
Citrate	Negative

Table 11: Biochemical reactions results of *Staphylococcus aureus* isolates

Characteristics	**Reaction**
Gram staining	$Gram^{+ve}$ cocci
Catalase	Positive
Oxidase	Negative
Coagulase test	Positive

Mannitol fermentation	Positive

Table 12: Biochemical reactions results of sucrose negative *Vibrio spp.* Isolates

Characteristics	**Reaction**
Gram staining	Gram $^{-ve}$, slightly curved rod
Catalase	Positive
Oxidase	Positive
TCBS agar	Green colony
Nitrate reduction	Positive
Hugh and Leifson's oxidative / fermentative metabolism	Positive

4.2 VITEK 2 Compact system confirmation

The presumptive bacterial isolates identified by biochemical tests were further confirmed by the VITEK 2 Compact system. The results of *E. coli and S. aureus* were confirmed by VITEK 2 and results are given in the tables.

Table 13: ***Escherichia coli* confirmation by VITEK2 compact system**

Well	**Test**	**Mnemonic**	**Reaction**
2	Ala-phe-pro-ARYLAMIDASE	APPA	-
3	ADONITOL	ADO	-
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	-
5	L-ARABITOL	IARL	-
7	D-CELLOBIOSE	dCEL	-

9	BETA-GALACTOSIDASE	BGAL	+
10	H2S PRODUCTION	H2S	-
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	-
12	Glutamyl arylamidase pNA	AGLTp	-
13	D-GLUCOSE	dGLU	+
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	-
15	FERMENTATION/GLUCOSE	OFF	+
17	BETA-GLUCOSIDASE	BGLU	-
18	D-MALTOSE	dMAL	-
19	D-MANNITOL	dMAN	+
20	D-MANNOSE	dMNE	+
21	BETA-XILOSIDASE	BXYL	-
22	BETA-Alanine arylamidase Pna	BAlap	-
23	L-Proline ARYLAMIDASE	ProA	-
26	LIPASE	LIP	-
27	PALATINOSE	PLE	-
29	Tyrosine ARYLAMIDASE	TyrA	-
31	UREASE	URE	-
32	D-SORBITAL	dSOR	+
33	SACCHAROSE/SUCROSE	SAC	+

34	D-TAGATOSE	dTAG	-
35	D-TREHALOSE	dTRE	+
36	CITRATE(SODIUM)	CIT	-
37	MALONATE	MNT	-
39	5-KETO-D-GLUCONATE	5KG	-
40	L-LACTATE alkalinisation	ILATk	-
41	ALPHA-GLUCOSIDASE	AGLU	-
42	SUCCINATE alkalinisation	SUCT	-
43	Beta-N-ACETYL-GALACTOSEAMINIDASE	NAGA	-
44	ALPA-GALACTOSIDASE	AGAL	-
45	PHOSPHATASE	PHOS	-
46	Glycine ARYLAMIDASE	GlyA	-
47	ORNITHINE DECARBOXYLASE	ODC	+
48	LYSINE DECARBOXYLASE	LDC	+
52	DECARBOXYLASE BASE	ODEC	N/A
53	L-HISTIDINE assimilation	IHISa	-
56	COUMARATE	CMT	+
57	BETA-GLUCORONIDASE	BGUR	+
58	O/129 RESISTANCE (comp.vibrio.)	O129R	+
59	Glu-Gly-Arg-ARYLANIDASE	GGAA	-

61	L-MAMATE assimilation	IMLTa	-
62	ELLMAN	ELLM	+
64	L-LACTATE assimilation	ILATa	-

Table 14: *Staphylococcus aureus* confirmed by VITEK 2 compact system

WELL	TEST	Mnemonic	Reaction
2	D-AMYGDALIN	AMY	-
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	PIPLC	-
5	D-XYLOSE	dXYL	-
8	ARGININE DIHYDROLASE 1	ADH 1	+
9	BETA-GALACTOSIDASE	BGAL	-
11	ALPHA-GLUCOSIDASE	AGLU	-
13	Ala-Phe-Pro ARYLAMIDASE	APPA	-
14	CYCLODEXTRIN	CDEX	-
15	L-Aspartate ARYLAMIDASE	AspA	-
16	BETA GALACTOPYRANOSIDASE	BGAR	-
17	ALPHA-MANNOSIDASE	AMAN	-
19	PHOSPHATASE	PHOS	+
20	Leucine ARYLAMIDASE	LeuA	-
23	L-Proline ARYLAMIDASE	ProA	-

24	BETA GLUCURONIDASE	BGURr	-
25	ALPHA-GALACTOSIDASE	AGAL	-
26	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	+
27	BETA-GLUCURONIDASE	BGUR	-
28	Alanine ARYLAMIDASE	AlaA	-
29	Tyrosine ARYLAMIDASE	TyrA	-
30	D-SORBITAL	dSOR	-
31	UREASE	URE	+
32	POLYMIXIN B RESISTANCE	POLYB	+
37	D-GALACTOSE	dGAL	+
38	D-RIBOSE	Drib	-
39	L-LACTATE alkalinisation	ILATk	+
42	LACTOSE	LAC	-
44	N-ACETYL-D-GLUCOSAMINE	NAG	-
45	D-MALTOSE	dMAL	+
46	BACITRACIN RESISTANCE	BACI	-
47	NOVOBIOCIN RESISTANCE	NOVO	-
50	GROWTH IN 6.5%NaCl	NC6.5	+
52	D-MANNITIOL	dMAN	+
53	D-MANNOSE	dMNE	+

54	METHYL-B-D-GLUCOPYRANOSIDE	MBdG	+
56	PULLULAN	PUL	-
57	D-RAFFINOSE	dRAF	+
58	O/129 RESISTANCE (comp.vibrio.)	O129R	+
59	SALICIN	SAL	-
60	SACCHAROSE/SUCROSE	SAC	+
62	D-TREHALOSE	dTRE	+
63	ARGININE DIHYDROLASE 2	ADH2	-
64	OPTOCHIN RESISTANCE	OPTO	-

4.3 Molecular confirmation of Vibrio spp.

After presumptive confirmation by biochemical tests, a total of 146 isolates tested for 16SrRNA, 138 isolated were found positive.

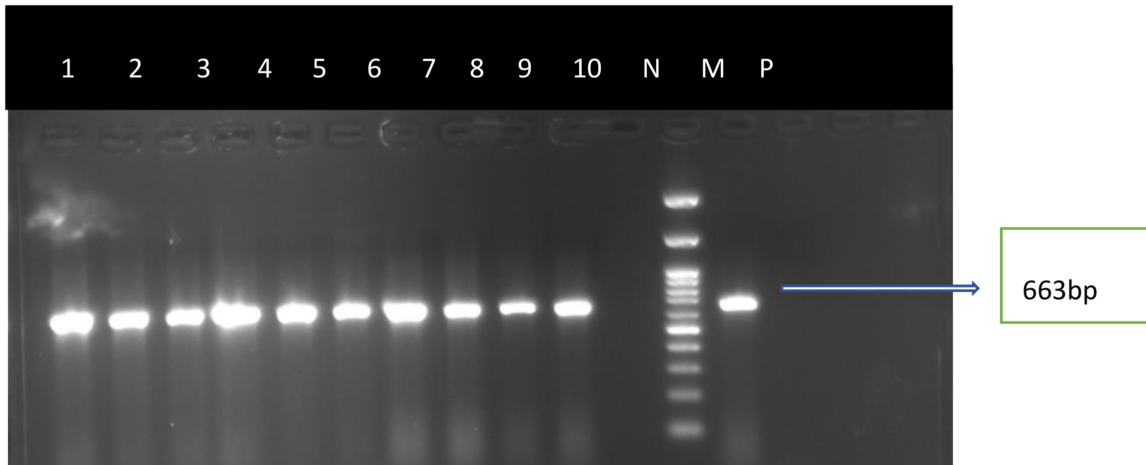

Fig 1: Amplification of 16SrRNA gene for the molecular confirmation of Vibrio isolates. Electrophoretic band patterns: Lane M – 100bp plus ladder,

Lane- 1,2,3,4,5,6,7,8,9,10– isolates positive for vibrio gene,

Lane P (+ve control),

Lane N- (-ve control).

Among 132 isolates, 113 isolates were confirmed as *E. coli*. Out of 127 presumptive isolates of *Staphylococcus aureus*, 62 isolates were confirmed and for *Vibrio spp.*, out of 144 isolates tested, 138 were confirmed. The confirmed isolates were tested for antimicrobial susceptibility and results of each bacterium was given below

4.4 Antimicrobial susceptibility of bacterial isolates

4.4.1 Antimicrobial susceptibility of *Escherichia coli*

A sum total of 113 isolates were tested for antimicrobial susceptibility. The analysis was done by using WHONET software. A total 19 antibiotics were used. Out of 113 isolates, 63 isolates were found resistant to at least one antibiotic tested, 28.5% (18 of 63) isolates displayed multidrug resistance (resistant to three or more than three antibiotics). As shown in the fig, maximum resistance was observed for Ampicillin (54 isolates, 47.8%) followed by Cefotaxime (23 isolates, 20.4%), Aztreonam (15 isolates, 13.3%), Erythromycin (8 isolates,7.1%), Ciprofloxacin (7 isolates, 6.2%), Ceftazidime (6 isolates, 5.3%), Nalidixic acid (5 isolates, 4.4%), Tetracycline (4 isolates, 3.6%), Cefoxitin (3 isolates, 2.7%) and Cefpodoxime (2 isolates, 1.8%). None of the isolates were found resistant to Trimethoprim/Sulfamethoxazole, Colistin, Chloramphenicol, and Amoxicillin/Clavulanic acid. Only 22 (19.4%) isolates were completely sensitive to all the antibiotics tested. The isolates exhibited intermediate resistance to AMP, CAZ, CRO, CTX, FEP, FOX, CPD, ATM, IPM, AMK, GEN, NAL, TCY, ERY, and CIP. The resistance rate to two drugs was highest at 38.09% (24 of 63 isolates), followed by resistance to one drug (33.3%, 21 of 63), three drugs (23.8%, 15 of 63) and four drugs (4.76%, 3 of 63)

Fig 2: Percentages of sensitivity/resistance to antibiotics of *E. coli* strains isolated from shrimp samples

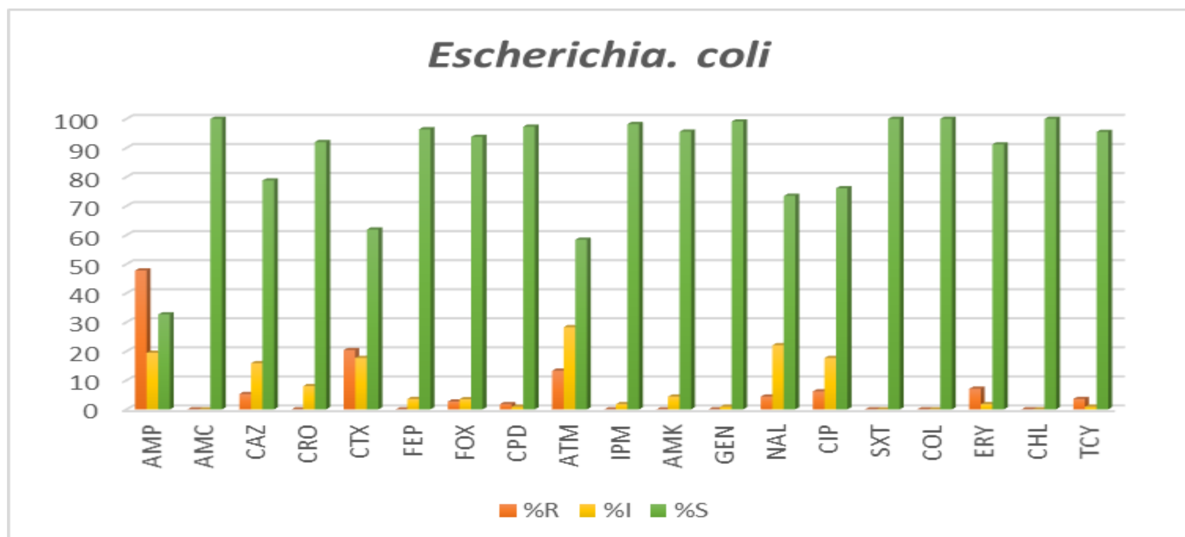

Table 15: The antibiotic resistant profile of *E. coli* isolates (n=113)

Antibiotic name	Code	%R	%I	%S
Ampicillin	AMP	47.8	19.5	32.7
Amoxicillin/Clavulanic acid	AMC	0	0	100
Ceftazidime	CAZ	5.3	15.9	78.8
Ceftriaxone	CRO	0	8	92
Cefotaxime	CTX	20.4	17.7	61.9
Cefepime	FEP	0	3.6	96.4
Cefoxitin	FOX	2.7	3.5	93.8
Cefpodoxime	CPD	1.8	0.9	97.3
Aztreonam	ATM	13.3	28.3	58.4
Imipenem	IPM	0	1.8	98.2
Amikacin	AMK	0	4.4	95.6
Gentamicin	GEN	0	0.9	99.1
Nalidixic acid	NAL	4.4	22.1	73.5
Ciprofloxacin	CIP	6.2	17.7	76.1
Trimethoprim/Sulfamethoxazole	SXT	0	0	100
Colistin	COL	0	0	100
Erythromycin	ERY	7.1	1.8	91.2
Chloramphenicol	CHL	0	0	100
Tetracycline	TCY	3.6	0.9	95.5

4.4.2 Percentage of antibiotic resistant, intermediate, and susceptible of *E. coli* isolated from various shrimp samples.

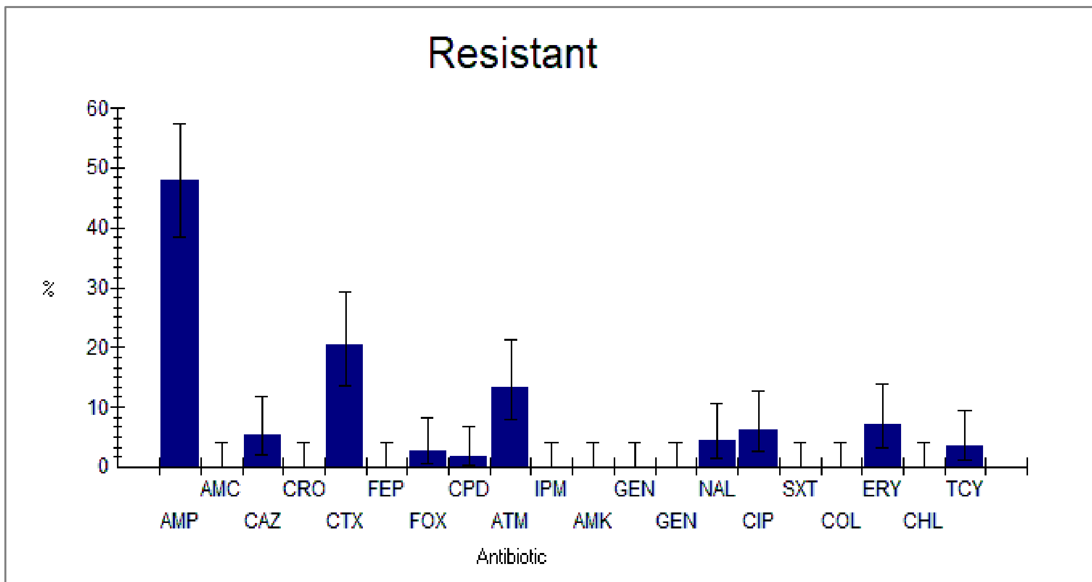

Fig 3: The order of antibiotic resistance shown among the E. coli isolates were AMP (47.8%)>CTX (20.4%)>ATM (13.3)>CIP (6.2%)>CAZ (5.3%)>NAL (4.4%)>FOX (2.7%)>CPD (1.8%)

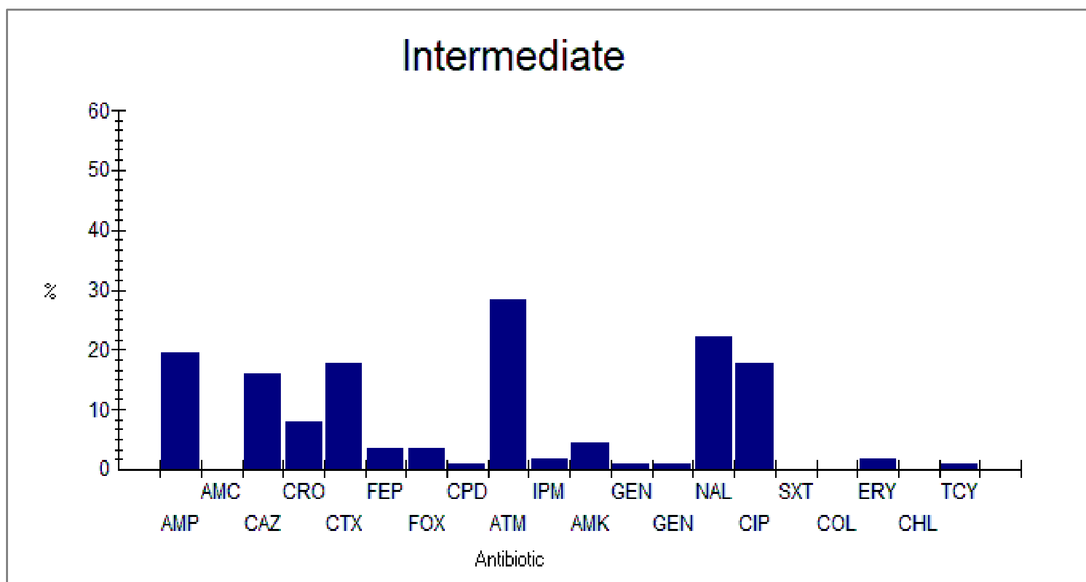

Fig 4: The order of intermediate resistance among the *E. coli* isolates were ATM (28.3%)>NAL (22.1%)>AMP (19.5%)>CTX=CIP (17.7%)>CAZ (15.9%)>CRO (8%)>AMK (4.4%)>FEP (3.6%)>FOX (3.5%)>IPM (1.8%)>CPD=GEN (0.9%).

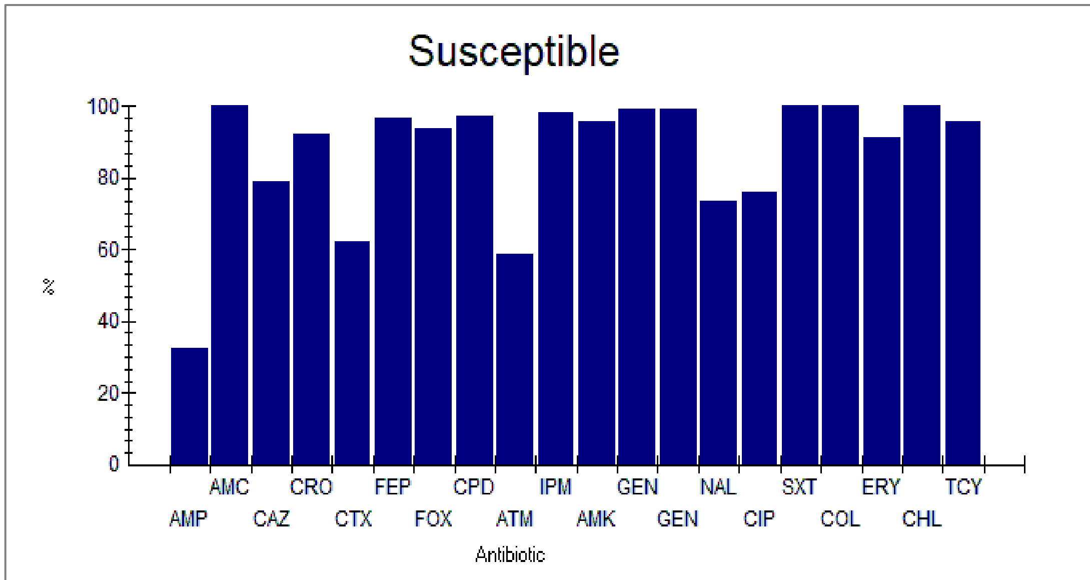

Fig 5: The order of antibiotic susceptibility among the *E. coli* isolates were AMC=SXT=COL=ERY=CHL=TCY(100%)>GEN (99.1%)>IPM (98.2%)>CPD (97.3%)>FEP (96.4%)>AMK (95.6%)>FOX (93.8%)>CRO (92%)>CAZ (78.8%)>CIP (76.1%)>NAL (73.5%)>CTX (61.9%)>ATM (58.4%)>AMP (32.7%).

4.4.3 Antimicrobial susceptibility of *Staphylococcus aureus*

A total of 62 isolates were tested for antimicrobial susceptibility. The analysis was done by using WHONET software. The bacteria were tested for susceptibility to sixteen antimicrobials representing twelve antimicrobial drug classes Among all the isolates tested, high resistance was showed to PEN (72.6%, 45 isolates), followed by AMP (43.5%, 27 isolates), ERY (16.4%, 0010 isolates), ATM (8.1%, five isolates), TCY (6.5%, four isolates), NAL (4.8%, three isolates).3.2% isolates were resistant to FEP, FOX, and IPM belonging to Cephalosporin IV class of Cephems, Cephamycin class of Cephems, and Carbapenems, respectively. None of the isolates were resistant to AMC, AMK, GEN, SXT, LNZ, and CHL. Intermediate resistance was showed to NAL=TCY (16.1%), ATM (14.5%), ERY (11.3%), CIP (9.8%), FEP (3.3%) and IPM (3.2%). 14.5 %, nine isolates displayed multidrug resistance to the antibiotics tested.

Fig 6: Percentages of sensitivity/resistance to antibiotics of *Staphylococcus aureus* strains isolated from shrimp samples

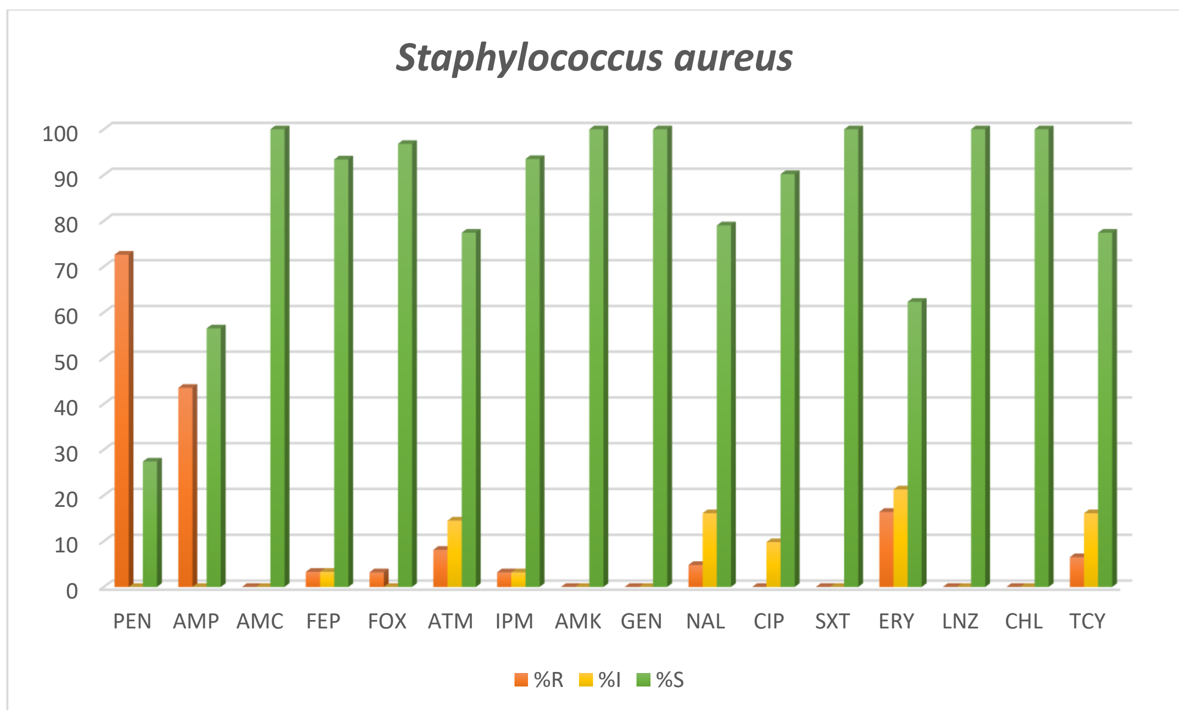

Table 16:The antibiotic resistant profile of *S. aureus* isolates (n=62)

Antibiotic name	Code	%R	%I	%S
Penicillin G	PEN	72.6	0	27.4
Ampicillin	AMP	43.5	0	56.5
Amoxicillin/Clavulanic acid	AMC	0	0	100
Cefepime	FEP	3.2	3.2	93.5
Cefoxitin	FOX	3.2	0	96.8
Aztreonam	ATM	8.1	14.5	77.4
Imipenem	IPM	3.2	3.2	93.5
Amikacin	AMK	0	0	100
Gentamicin	GEN	0	0	100
Nalidixic acid	NAL	4.8	16.1	79
Ciprofloxacin	CIP	0	9.8	90.2
Trimethoprim/Sulfamethoxazole	SXT	0	0	100
Erythromycin	ERY	16.4	21.3	62.3
Linezolid	LNZ	0	0	100
Chloramphenicol	CHL	0	0	100
Tetracycline	TCY	6.5	16.1	77.4

4.4.4 Percentage of antibiotic resistant, intermediate, and susceptibility of *Staphylococcus aureus* isolated from various shrimp samples.

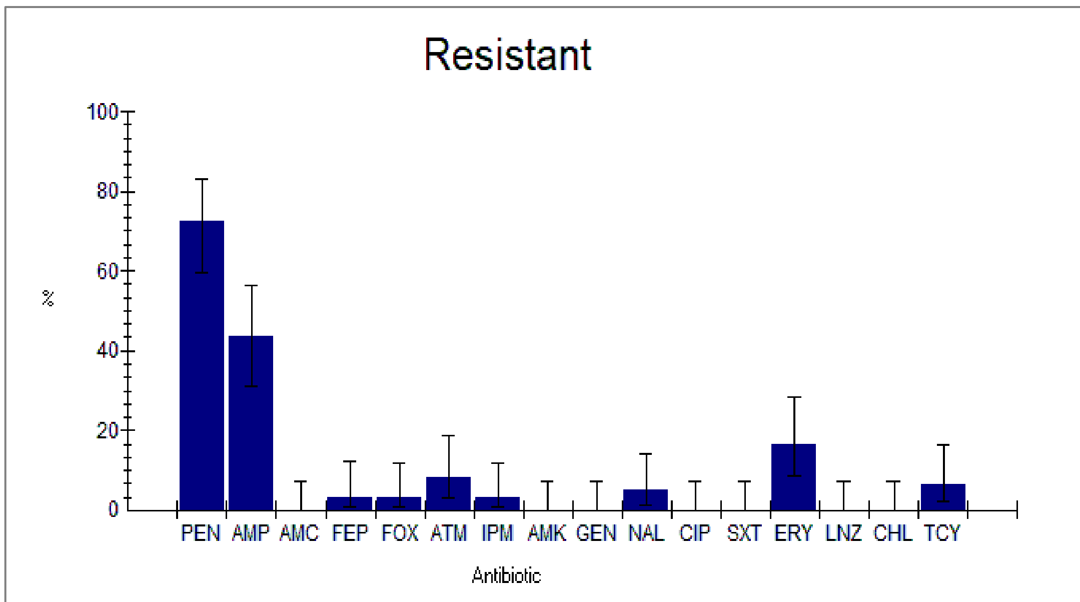

Fig 7: The order of antibiotic resistance shown among the *Staphylococcus aureus* isolates were PEN (72.6%)>AMP (43.5%)>ERY (16.4%)>ATM (8.1%)>TCY(6.1%)>NAL (4.8%)>FEP=FOX=IPM (3.2%).

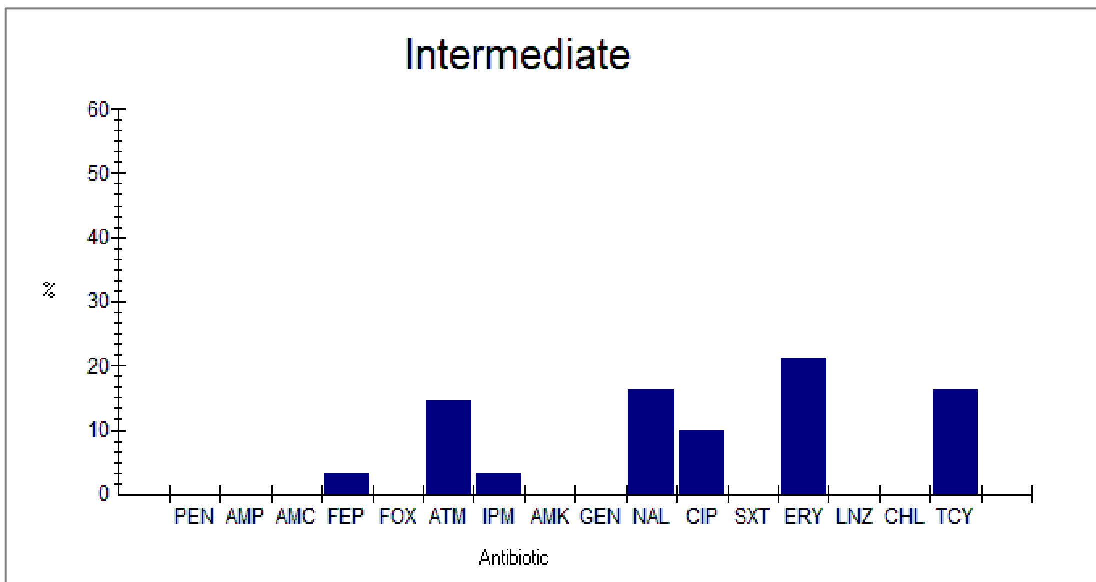

Fig 8: The order of intermediate resistance rates among the *Staphylococcus aureus* isolates were ERY (21.3%)>NAL=TCY (16.1%)>ATM (14.5%)>CIP (9.8%)>FEP =IPM (3.2%).

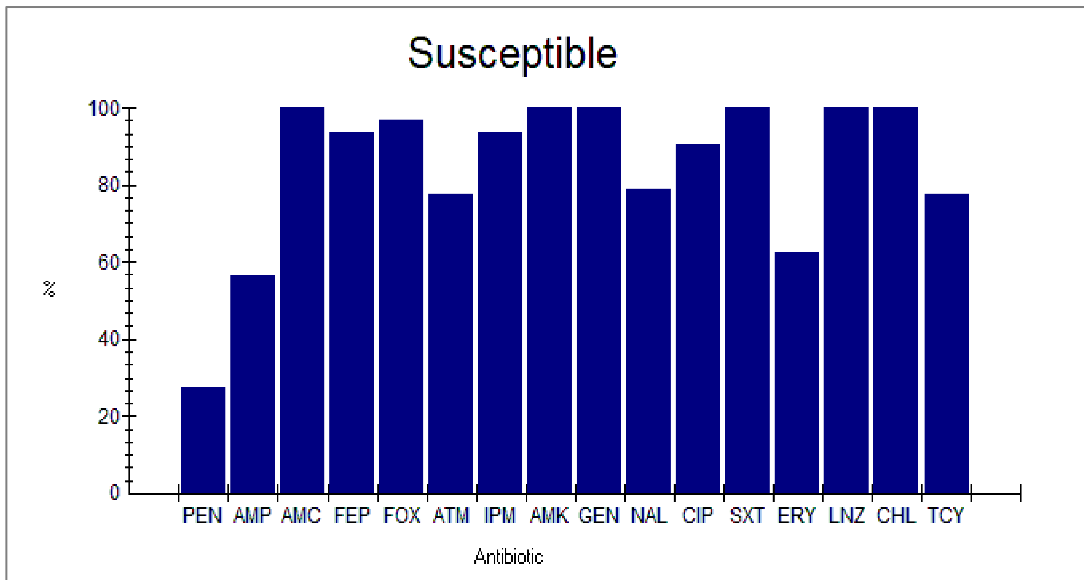

Fig 9: The order of antibiotic susceptibility among the *Staphylococcus aureus* isolates were AMC=AMK=GEN=SXT=LNZ=CHL (100%)>FOX (96.8%)>IPM=FEP (93.5%)>CIP (90.2%)>NAL (79%)>ATM=TCY (77.4%)>ERY (62.3%)>AMP(56.5%)>PEN (27.4%).

4.4.5 Antimicrobial susceptibility of *Vibrio spp*.

A total of 138 Vibrio isolates were tested for their susceptibility to antimicrobial agents. The bacteria were tested for susceptibility to sixteen antimicrobials representing ten antimicrobial drug classes. Among the 138 Vibrio isolates, 65 isolates (47.1%) showed resistance to at least one of the sixteen tested antibiotics, and 29 (21.01%) isolates were sensitive to all the antibiotics tested. Nineteen isolates (13.7%) displayed multidrug resistance (resistant to three or more than three antibiotic drugs). Maximum resistance was observed against the AMP (53 isolates, 38.4%),followed by ERY (23 isolates, 16.7%), AMK (17 isolates,12.3%), ATM (9 isolates, 6.5%), CTX (8 isolates, 5.8%), , CAZ & TCY (5 isolates, 3.6%), FOX (3 isolates, 2.2%) and CIP (1 isolate, 0.7%). A high incidence of intermediate resistance was observed against Penicillin class of antibiotic Ampicillin (22.5%) and Quinolone class of antibiotic Nalidixic acid (16.7%). Complete susceptibility was seen to antibiotics like IPM and MEM belonging to carbapenem class and also to CHL and SXT neither showing intermediate resistance nor resistance.

Fig 10: Percentages of sensitivity/resistance to antibiotics of *Vibrio spp*. isolated from shrimp sample

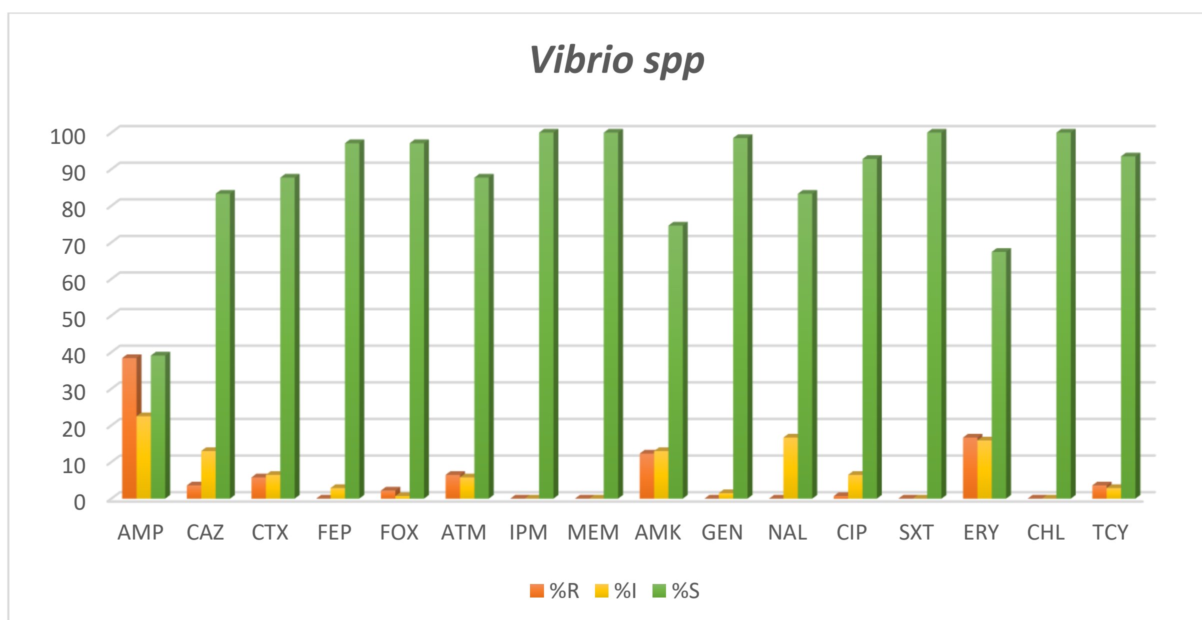

Table 17: The antibiotic resistant profile of *Vibrio* isolates (n=138)

Code	Antibiotic name	%R	%I	%S
AMP	Ampicillin	38.4	22.5	39.1
CAZ	Ceftazidime	3.6	13	83.3
CTX	Cefotaxime	5.8	6.5	87.7
FEP	Cefepime	0	2.9	97.1
FOX	Cefoxitin	2.2	0.7	97.1
ATM	Aztreonam	6.5	5.8	87.7
IPM	Imipenem	0	0	100
MEM	Meropenem	0	0	100
AMK	Amikacin	12.3	13	74.6
GEN	Gentamicin	0	1.5	98.5
NAL	Nalidixic acid	0	16.7	83.3
CIP	Ciprofloxacin	0.7	6.5	92.8
SXT	Trimethoprim/Sulfamethoxazole	0	0	100
ERY	Erythromycin	16.7	15.9	67.4
CHL	Chloramphenicol	0	0	100
TCY	Tetracycline	3.6	2.9	93.5

4.4.6 Percentage of antibiotic resistant, intermediate, and susceptibility of *Vibrio spp.* isolated from various shrimp samples.

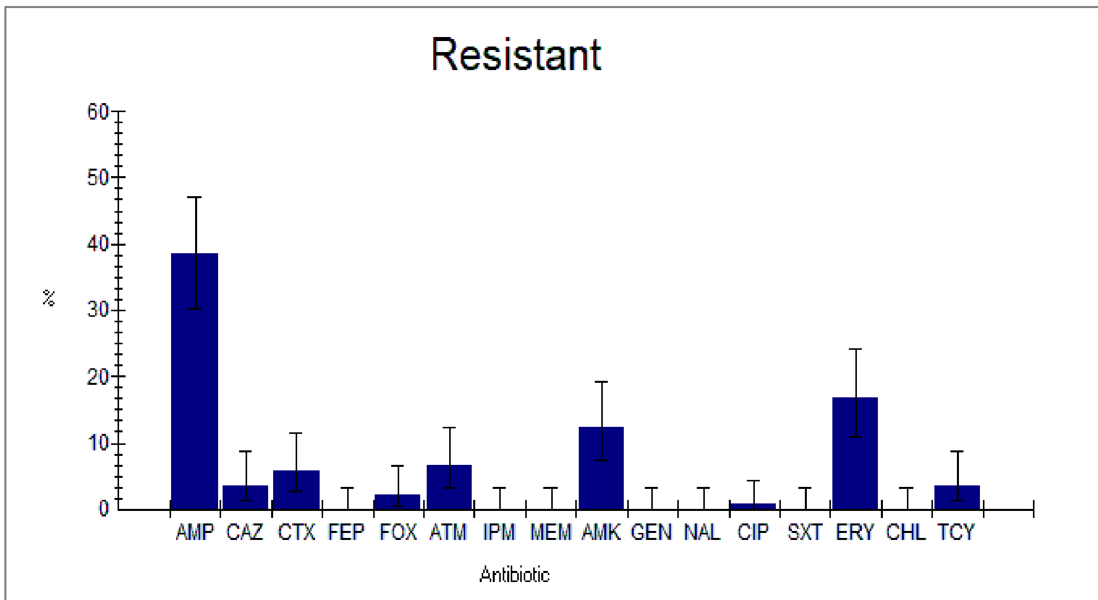

Fig 11: The order of antibiotic resistance among the *Vibrio* isolates were AMP (38.4%)> ERY (16.7%)>AMK (12.3%)> ATM (6.5%)>CTX (5.8%)> (5.1%)>CAZ & TCY(3.6%)>FOX(2.2%)>CIP (0.7%).

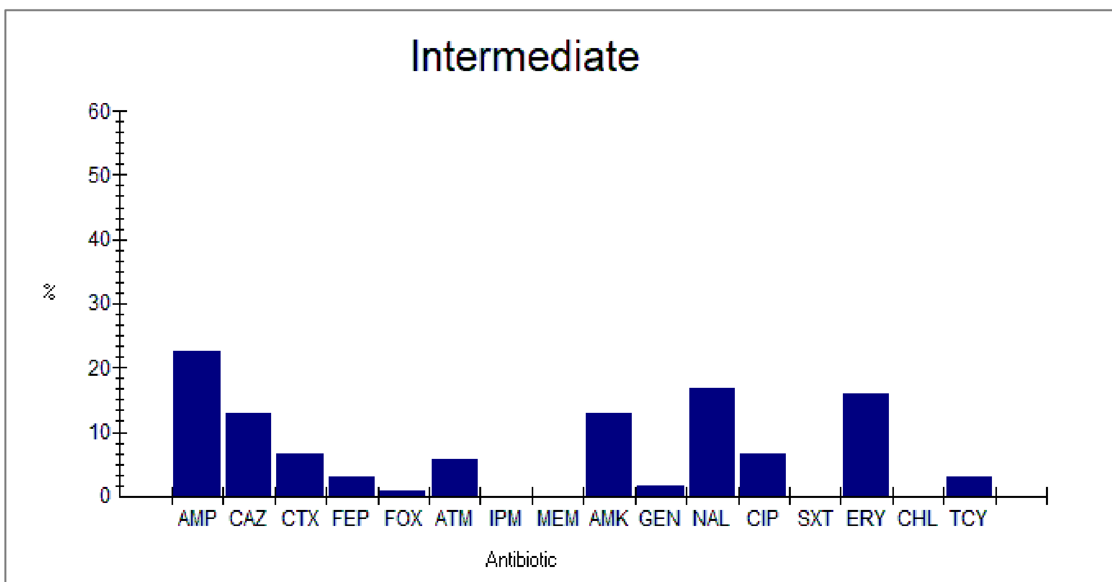

Fig 12: The order of intermediate resistance among the *Vibrio* isolates were AMP (23.9%)>NAL (16.7%)>CAZ (13%)>AMK (12.3%)>CIP (6.5%)>CTX (5.8%)>ATM (5.1%)>FEP (2.9%)>FOX (0.7%).

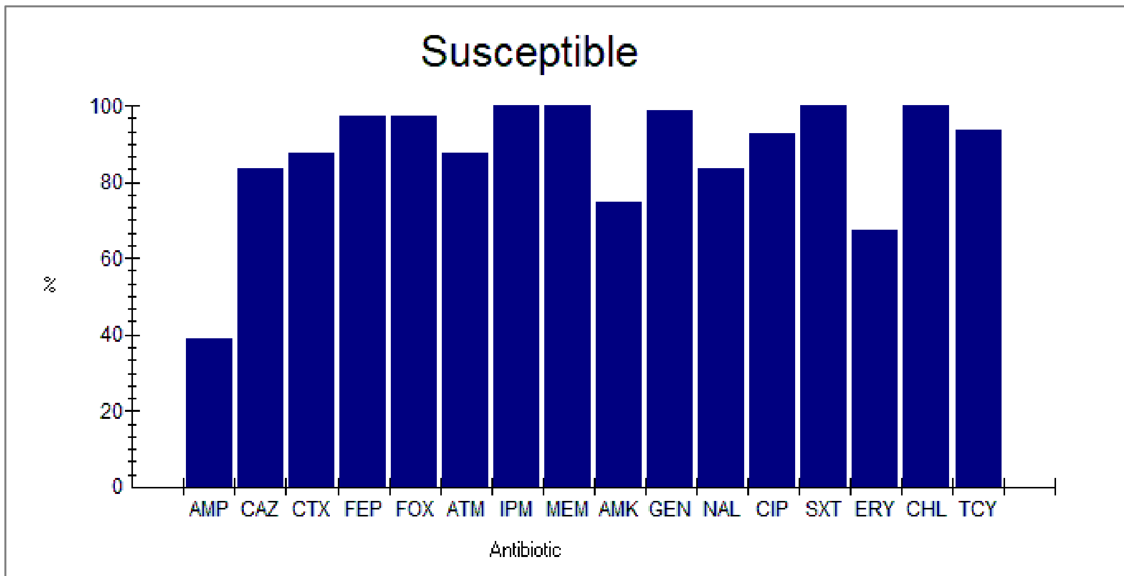

Fig 13: The order of antibiotic susceptibility among the *Vibrio* isolates were IPM=MEM=GEN=SXT=TCY (100%)>FOX (98.6%)>CHL(97.8%)>FEP (97.1%)>CIP (92.8)>ATM (89.9%)>CTX (88.4%)>CAZ =NAL(83.3%)>AMK (75.4%)>AMP (41.3%)

4.5 Molecular characterisation of AMR

4.5.1 PCR amplification of blaTEM gene

Isolates of *E. coli*, *Staphylococcus aureus* and *Vibrio spp*. were mostly resistant to the β-lactam class of antibiotics. Most of the β-lactam resistant isolates are known to carry out the bla-TEM gene, so the prevalence of the bla-TEM gene was tested using PCR. A total of 134 isolates exhibited resistance against ampicillin along with Aztreonam and Cefotaxime, β- lactam class of antibiotics. The isolates were tested for the occurrence of bla-TEM gene using TEM-F and TEM-R primers, which yielded an amplicon size of 800bp. Out of 134 isolates tested, 102 were positive for the bla-TEM gene. The high level (76.11 %) of prevalence was shown in the fig 14.

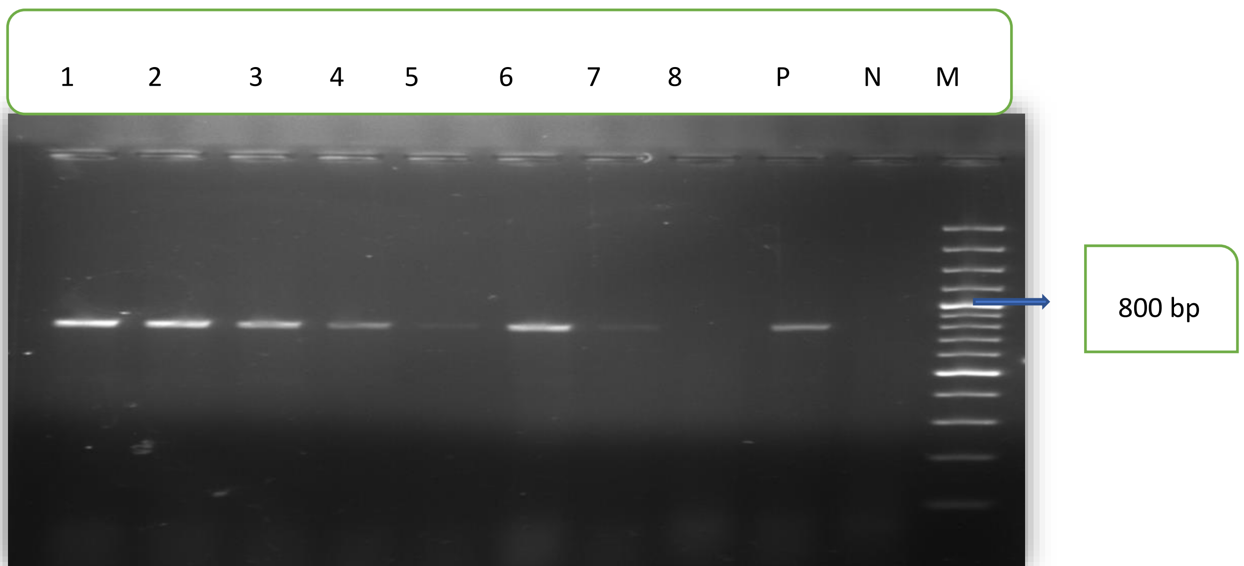

Fig14: PCR amplification of bla-TEM gene.

Lane M- 100 bp ladder

Lane 1-7- positive samples

Lane 8- negative sample

Lane P- (+ve control)

Lane N - (-ve control)

DISCUSSION

5. DISCUSSION

5.1 Prevalence of microflora in shrimp samples

In the present study, the prevalence of *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio spp* was observed. Coliforms belonging to the Enterobacteriaceae family were dominant seafood bacteria, among which *E. coli* was found frequently in contaminated seafood samples. According to Chakravarty *et al*., 2015, they observed the occurrence of *Escherichia coli* in the shrimp, fish, mutton, and chicken meat samples. In the current study, a total of 113 isolates were identified as *Escherichia coli* isolated from the whole shrimp sample. The bacteria enriched in EC broth showed gas production in Durham tubes were taken as positive for *E. coli*. On L-EMB agar plates, inoculated bacteria formed dark centered and flat colonies with a green metallic sheen, and these were identified as *E. coli*. This was in accordance with the findings of Zinnah *et al*., 2007, as they also identified and characterized *E. coli* bacteria on L-EMB agar with dark centered and flat, green metallic sheen colonies with the production of gas in EC broth indicating *E. coli* as lactose fermentative bacteria. In the present study, the biochemical characteristics have revealed that these bacteria were gram-negative rods, oxidase negative, and catalase-positive with the IMViC pattern of (++--). These findings were similar to the reports of Thampuran *et al.,* 2005, who determined the IMViC pattern for 484 presumptive *E. coli* isolates of finfish samples in and around Cochin, India. Most of the isolates were biotype I (+ + - -), and this was similar to that of isolates of the present study. According to Roven and Levin (1970) report, *E. coli* was phenotypically identified by the Indole, Methyl red, Voges-Proskauer, and Citrate (IMViC) test reactions (- - + -) which differ from that of IMViC results of *E. coli* isolated in this study.

According to Arfatahery *et al.,* 2015, the prevalence of *Staphylococcus aureus* in marine and farmed shrimps was (24.6%). Incidence of *S. aureus* (20%) in freshwater fish and seafood harvested in the southern region of Brazil (Ayulo *et al.,* 1994). *Staphylococci*, which are found in higher animals, are not naturally associated with the aquatic environment (Huss, 1988). Their presence in fish and seafood is

solely due to secondary contamination during handling (Huss, 1988; Austin, 2007). Therefore, *Staphylococcus* is an indicator of hygiene, and their presence in fish reflects unhygienic handling.

In the present study, a total of 52 live shrimp from 26 different farms were tested for *S. aureus* prevalence. A total of 127 bacterial strains were isolated and characterized. Circular, smooth, convex, moist, 2-3 mm in diameter, grey to jet-black, frequently with light-colored (off-white) margin surrounded by opaque zone and often with outer clear zone colonies were formed on the BP agar plates, and these were presumptively considered as *Staphylococcus aureus*. These were gram-positive cocci, oxidase negative, catalase and coagulase-positive and mannitol fermentative. Out of 127, 62 isolates were identified as coagulase-positive *S. aureus* and the remaining isolates as coagulase-negative *Staphylococci.* This was supported by the findings of Buller, 2004 who reported, shiny black colonies, frequently with light colored (off- white) margin, surrounded by opaque zone showed bright yellow colonies on Mannitol salt agar, which indicated positive for MSA test and coagulase-positive were identified as *S. aureus*. Baird Parker, 1962 was the first person to report Baird-Parker agar as a selective medium for the enumeration of *Staphylococcus aureus* in foods. Moreover, these species on BP medium forms as grey to black or brown-grey colonies because of tellurite reduction.

As per the reports of Gomez-Gil *et al*., 1998 and Zhang *et al*., 2014, *Vibrio spp* were predominantly found in the shrimp digestive tract. In this study, a total of 138 sucrose negative *Vibrio spp* were identified from different farms. These bacteria were isolated from TCBS agar plates as green or greenish-blue opaque colonies and considered as sucrose negative *Vibrio spp*. These findings were supported by Kobayashi *et al.,* 1963 in which they reported TCBS as the selective and differentiating medium for sucrose and non-sucrose *Vibrio spp.*

In the present study, further confirmation of bacterial isolates was done by VITEK 2 COMPACT system by gram-positive and gram-negative reagent cards. The test results of *E. coli* were confirmed by gram-negative card whereas *S. aureus* by gram-positive card. This was supported by the findings of Renaud *et al.*, 2005, which confirmed most of the gram-negative bacteria by the VITEK 2 system. Spanu

et al., 2003, used VITEK2 for the confirmation of *Staphylococcus species* and suggested that the VITEK 2 system can provide rapid, reliable, and accurate species-level identification of *Staphylococci* responsible for bloodstream infections. In this study, the *Vibrio* genus was confirmed by 16SrRNA performing polymerase chain reaction, which was, according to Tarr *et al.,* 2007.

5.2 Antibiotic susceptibility associated with the isolates

Antibiotics used in the study were Ampicillin, Amoxicillin-clavulanic acid, Amikacin, Aztreonam, Chloramphenicol, Cefoxitin, Cefpodoxime, Ceftriaxone, Ciprofloxacin, Erythromycin, Gentamycin, Nalidixic acid, Tetracycline, Trimethoprim-sulfamethoxazole were commonly used antibiotics in shrimp and fish farming (Vivekanandhan *et al*., 2002; Holmstrom *et al.,* 2003; Belém-Costa & Cyrino, 2006; Bermúdez -Almada & Plascencia , 2012; Costa *et al*., 2015) and human medicine (Rhodes *et al*., 2000; MacMillan, 2001).

A total of 113 *E. coli* isolates were tested for antimicrobial susceptibility. Out of 113, 63 isolates showed resistance to at least one antibiotic tested. Maximum resistance was observed for Ampicillin (54 isolates, 47.8%) followed by Cefotaxime (23 isolates, 20.4%), Aztreonam (15 isolates, 13.3%), Erythromycin (8 isolates,7.1%), Ciprofloxacin (7 isolates, 6.2%), Ceftazidime (6 isolates, 5.3%), Nalidixic acid (5 isolates, 4.4%), Tetracycline (4 isolates, 3.6%), Cefoxitin (3 isolates, 2.7%) and Cefpodoxime (2 isolates, 1.8%). These findings were in agreement with the report of data from many previous studies which found that *E. coli* from food animals exhibited common resistance to Tetracycline and Ampicillin (Schroeder *et al*., 2003; Srinivasan *et al*., 2007; Van *et al*., 2008; Vasilakopoulou *et al*., 2009)

Cheng *et al.,* 2019 investigated the antibiotic susceptibility profiles of *Escherichia coli* isolated from *Penaeus vannamei* and observed that these isolates are commonly resistant to β- lactam antibiotics except IMP, which was analogous with this study. Tricia *et al.,*2006 reported that *E. coli* isolates showed 43% antibiotic resistance to Ampicillin and sensitivity towards gentamicin, which was similar to this study. Van *et al.,* 2008 who reported 56.25% resistant to Ampicillin and 63% resistance to Erythromycin, which was in contrast with this study in which a high incidence of Ampicillin resistance was observed than the Erythromycin. This might

reflect the difference in the resistance pattern of antimicrobial agents in different regions.

In this present study, none of the isolates were found resistant to Trimethoprim/Sulfamethoxazole, Colistin, Chloramphenicol, and Amoxicillin/Clavulanic acid. This indicates β-lactam antibiotic, along with its inhibitor (Amoxicillin/Clavulanic acid), can be used to treat infections caused by *E. coli.* However, Tuo *et al.,* 2018 reported a low level of incidence of resistance (5.8%) against Amoxicillin/Clavulanic acid, but the high incidence of resistance which was about 80% to 100% against Amoxicillin/Clavulanic in aquaculture was reported by Jacobs & Chenia, 2007. The resistance rate of two drugs was highest at 38.09% (24 of 63 isolates), followed by resistance to one drug (33.3%, 21 of 63), three drugs (23.8%, 15 of 63) and four drugs (4.76%, 3 of 63) which was parallel with the reports of Changkaew *et al.,* 2014 who characterized antibiotic resistance in *E.coli* isolated from shrimp and its environment.

A total of 62 isolates of *Staphylococcus aureus* were tested for their antibiotic susceptibility to different antibiotics. Resistance to the penicillinase-stable Penicillin (cefoxitin) is referred to as methicillin resistance (MRSA) or oxacillin resistance (Dunman *et al.,* 2001). Cefoxitin resistance is used as a surrogate marker for the detection of methicillin-resistant *Staphylococcus aureus* (Fernande *et al.,* 2005). In the present study, high resistance was showed to PEN (72.6%), followed by AMP (43.5%), ERY (16.4%), ATM (8.1%), TCY (6.5%), NAL (4.8%). The occurrence of high resistance towards the β-lactam class of antibiotics followed by macrolides. In this study, Penicillin displayed high resistance of 72.6% which was higher than the previous study which reported 56%(PEN) for the imported shrimp in Switzerland (Boss *et al*., 2016) and lower for the *Staphylococcus aureus* from aquatic products in China (Rong *et al*., 2017) and isolates from marine and farmed shrimps of Iran (Arfatahery *et al.,* 2015)

Although Chloramphenicol is an older generation drug, all the strains of *Staphylococcus aureus* were found to be sensitive. This may be due to the rare application of Chloramphenicol in disease treatment because of its toxicity; this was

supported by Visnuvinayagam *et al*., 2013 who reported that 154 isolates of *S. aureus* from 11 fish farms showed susceptibility to Chloramphenicol.

The incidence of MRSA in raw fish and shellfish was ranging from 6-11% as per the report of Vaiyapuri *et al*., 2019. Out of 105 fish samples collected from Cochin and Mumbai coasts, exhibited a low prevalence of MRSA, i.e., MRSA was found only in one sample. (Visnuvinayagam *et al*., 2015) which is similar to this study, where it was only 3.2% of isolates showed resistance to cefoxitin. According to Hammad *et al.,* 2012, a high prevalence of MRSA strains has been reported from Japanese retail ready to eat raw fish, whereas Pesavento *et al.,* 2007 have reported a low isolation rate of MRSA strains from raw pork and beef. The first report of MRSA from cage cultured tilapia disclosed a high resistance rate of 50% MRSA (Atyah et al., 2010) and incidence of 50% MRSA from chicken samples in Korean slaughterhouses as per the report of Kwon *et al.,* 2006. Though comparisons among different sample types and geographical regions on the isolation rates of MRSA cannot be made, it is compelling to state the seafood, and their products do harbor MRSA.

In the present study, a total of 138 *Vibrio* isolates were tested for their susceptibility to antimicrobial agents. Among the 138 *Vibrio* isolates, 65 isolates (47.1%) showed resistance to at least one of the sixteen tested antibiotics, and 29 (21.01%) isolates were sensitive to all the antibiotics tested. Maximum resistance was observed against the AMP (53 isolates, 38.4%),followed by ERY (23 isolates, 16.7%), AMK (17 isolates,12.3%), ATM (9 isolates, 6.5%), CTX (8 isolates, 5.8%), , CAZ & TCY (5 isolates, 3.6%), FOX (3 isolates, 2.2%) and CIP (1 isolate, 0.7%). Sirikorn *et al.,* 2010 and Rosa *et al*., 2010 reported that *Vibrio spp* isolated from *Penaeus monodon* exhibited resistance to Ampicillin (61%) and Erythromycin (20%) which was accordance with this study in which maximum resistance was showed against Ampicillin followed by Erythromycin and susceptibility towards Chloramphenicol. 53% of *Vibrio spp* were found resistant against Ampicillin, and it was reported to be less effective against *Vibrio spp* by Costa *et al.,* 2005.

Complete susceptibility was seen to antibiotics like IPM and MEM belonging to carbapenem class and also to CHL and SXT, neither showing

intermediate resistance nor resistance. None of the isolates were found resistant to Nalidixic acid which was at par with the study of Rosa *et al.,* 2009 who reported complete sensitivity of *Vibrio spp* towards Nalidixic acid and contrast with the study of Lim *et al.,* 2013 in which *V. parahaemolyticus* showed 60% resistance to Nalidixic acid. *V. parahaemolyticus* showed susceptibility against Chloramphenicol, Nalidixic acid, Imipenem, Trimethoprim-sulfamethoxazole (Ottaviani *et al*., 2001; Devi *et al.,* 2009; Shaw *et al.,*2014). Ampicillin resistance was most repeatedly detected among *Vibrio species* isolated from shrimp farms (Vaseeharan *et al*., 2005) and hatcheries (Hameed *et al.,* 2003) as well as from fishery products (Akinbowale *et al.,* 2006).

5.3 Characterisation of Antimicrobial Resistance Genes (ARG's)

Antimicrobial Resistance Genes (ARG's) may have evolved naturally or by abuse of antibiotics in human and animal medicines, which has led to the selection and spread of resistant genes. ARG's were responsible for the bacterial resistance against the antibiotics used. This ARG's can be intrinsically present or acquired through horizontal gene transfer. All the three bacterial isolates showed resistance towards Ampicillin, although it is not commonly used in shrimp culture. Hence, this may be attributed to intrinsic resistance. BlaTEM gene was commonly present ARG responsible for Ampicillin resistance, as reported by Olesen *et al*., 2004 and Delmani *et al*.,2017, which can be compared with this study. TEM- type β-lactamases were the most prevalent in ampicillin resistant *E. coli* isolates from food and food-producing animals (Brinas *et al*., 2002; Guerra *et al.,* 2003; Jouini *et al.,* 2009; Van *et al*., 2008) which can be related to this study. *Vibrio spp* isolated from river and aquaculture water sources showed amplification of bla TEM gene (Chikwendu *et al*., 2014) which can be compared to this study. Detection of the blaTEM gene in *Staphylococcus aureus* was reported by Xu *et al*., 2014 from food isolates, which can be analogous with this study. Of the total 134 isolates tested, 102 were positive for the bla-TEM gene, which indicated a high level (76.1%) of prevalence, which was supported with the findings of Weill *et al*., 2004. 80.9% of the isolates harbor a blaTEM gene for the TEM type enzyme in the family of Enterobacteriaceae, according to the report of Amador *et al.,* 2009.

SUMMARY

&

CONCLUSION

6. SUMMARY AND CONCLUSION

The shrimp farming industry has a tremendous commercial significance in Indian aquaculture. However, the intensified and accelerated growth of this activity has resulted in the outbreaks of devastating diseases. The indiscriminate use of antibiotics to minimize the potential adverse effects of diseases results in the development of resistance by the cultured shrimps' microflora. The drug-resistant bacteria may disseminate their resistant genes through horizontal gene transfer and may reach humans through the food chain. This study focused on the antimicrobial resistance among *Escherichia coli, Staphylococcus aureus, and Vibrio spp.* A total of 156 cultured shrimps were collected from 26 different farms of Maharashtra and Gujarat. They were dissected and processed aseptically, and a total of 113 isolates of *E. coli*, 62 isolates of *Staphylococcus aureus*, and 138 isolates of *Vibrio spp* were identified and characterized based on standard microbiological techniques. These isolates were tested to antimicrobial susceptibility, as proposed by Kirby and Bauer (1966). The antibiotics used in the study were based on the CSLI standards.

Out of 113 isolates of *E. coli* tested for antimicrobial susceptibility. The pattern of resistance shown by isolates as follows AMP (47.8%)>CTX (20.4%)>ATM (13.3)>CIP (6.2%)>CAZ (5.3%)>NAL (4.4%)>FOX (2.7%)>CPD (1.8%) and the pattern of sensitivity as follows AMC=SXT=COL=ERY=CHL=TCY(100%)>GEN (99.1%)>IPM (98.2%)>CPD (97.3%)>FEP (96.4%)>AMK (95.6%)>FOX (93.8%)>CRO (92%)>CAZ (78.8%)>CIP (76.1%)>NAL (73.5%)>CTX (61.9%)>ATM (58.4%)>AMP (32.7%). High prevalence of resistance showed by β-lactam class of antibiotics.

Of the 62 isolates of *Staphylococcus aureus* tested against different antibiotics revealed the order of antibiotic resistance pattern of PEN (72.6%)>AMP (43.5%)>ERY (16.4%)>ATM (8.1%)>TCY (6.1%)>NAL (4.8%)>FEP=FOX=IPM (3.2%). The susceptibility of the isolates was in the order of AMC=AMK=GEN=SXT=LNZ=CHL (100%)>FOX (96.8%)>IPM=FEP (93.5%)>CIP (90.2%)>NAL (79%)>ATM=TCY (77.4%)>ERY (62.3%)>AMP (56.5%)>PEN (27.4%).

A total of 138 isolates were tested for antibiotic susceptibility and the pattern of antibiotic resistance was as follows AMP (38.4%)> ERY (16.7%)>AMK (12.3%)> ATM (6.5%)>CTX (5.8%)> (5.1%)>CAZ & TCY (3.6%)>FOX (2.2%)>CIP (0.7%). The sensitivity pattern was in the order of IPM=MEM=GEN=SXT=TCY (100%)>FOX (98.6%)>CHL (97.8%)>FEP (97.1%)>CIP (92.8)>ATM (89.9%)>CTX (88.4%)>CAZ =NAL (83.3%)>AMK (75.4%)>AMP (41.3%). High prevalence of resistance showed by β-lactam class of antibiotics. Multi drug resistance displayed by *E. coli, Staphylococcus aureus* and *Vibrio spp* was 18 isolates, 9 isolates and 19 isolates respectively.

Molecular characterization of the most prevalent resistance was performed using PCR. Among the resistance pattern, ampicillin resistance was predominant at a rate of 76.1%. Along with ampicillin resistance, several isolates showed resistance to Aztreonam and Cefotaxime. PCR screening was done for the ampicillin-resistant isolates for the presence of the blaTEM gene, a common gene responsible for ampicillin resistance.

Hence, the study could provide the basic information regarding *Escherichia coli*, *Staphylococcus aureus* and *Vibrio spp* of cultured shrimps, which could be vital for creating a data baseline for which further research could explore their possible application as probiotics and could provide the knowledge about the antimicrobial resistance associated with the isolates that could help in understanding the propagation of AMR and in formulating mitigating measures.

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7.REFERENCES

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APPENDIX

ABBREVIATIONS

min	: minute
sec	: second
gm	: gram
ppt	: Parts per thousand
L	: litre
kg	: kilogram
hr	: hour
BP	: Baird Parker
TCBS	: Thiosulfate citrate bile salts sucrose
MHA	: Mueller Hinton Agar
MSA	: Mannitol salt agar
MR	: Methyl red
VP	: Voges-Proskauer
H_20_2	: Hydrogen peroxide
EDTA	: Ethylene Diamine Tetra- acetic acid
TE	: Tris-EDTA
CLSI	: Clinical Laboratory and Standards Institute
AMR	: Antimicrobial resistance
PCR	: Polymerase Chain Reaction
EC broth	: *Escherichia coli* broth
PBS	: Phosphate buffered saline

L-EMB	: Levine-Eosine Methylene Blue
FEP	: Cefepime
FOX	: Cefoxitin
ATM	: Aztreonam
AMC	: Amoxicillin/Clavulanic acid
AMK	: Amikacin
CHL	: Chloramphenicol
TCY	: Tetracycline
dil	: dilute
HCL	: Hydrogen chloride
SXT	: Trimethoprim/Sulfamethoxazole
DNA	: Deoxyribose nucleic acid
ml	: millilitre
mg	: milligram
NaCl	: Sodium chloride

APPENDIX-I

Apparatus, Materials and Solutions

Equipments

- Autoclave (Tomy sx-700, USA)
- Centrifuge
- Deep Freezer (-20^{0}C) (Remi Industries, India)
- Electronic balance (Denver Industries, USA)
- Gel documentation system (Aplegen, USA)
- Horizontal Electrophoresis assembly (Bangalore GeneiPvt.Ltd., India)
- Laminar flow (Yorko scientific Industries, India)
- Micropipettes (Eppendorf, Germany; Tarsons, India)
- Microwaveoven (Duracef, USA)
- PCR work station
- UV transilluminator (Manisha Enterprise, India)
- Water bath (Julabo, Germany)

Consumables and disposables

- Chemicals (HiMedia Laboratories, Mumbai)
- Glassware (Borosil, India)
- Molecular weight markers (Fermentas, USA)
- PCR reagents (ThermoFisher Scientific, USA)
- Plasticware (TarsonsPvt.Ltd., India)
- Primers (Bioinnovations, Mumbai)

APPENDIX-II

Reagent composition

1.Bacterial culture

1.1Baird-Parker Medium

A) Basal medium

Ingredients	Gm/litre
Tryptone	10.000
Beef extract	5.000
Yeast extract	1.000
Sodium pyruvate	12.000
Glycine	12.000
LiCl.6H2O	5.000
Agar	15.000

B) Before pouring into the plates, add the following per 100ml medium,

i) Sterile egg yolk: 5ml of 50% egg yolk

ii) Sterile potassium tellurite: 1ml of 1%solution

1.1 Eosin Methylene Blue Agar (EMB)

Ingredients	Gm/litre
Peptic digest of animal tissue	10.000
Dipotassium phosphate	2.000
Lactose	10.000
Eosin - Y	0.400
Methylene blue	0.065
Agar	15.000

1.2 Thiosulfate citrate bile salts sucrose (TCBS) Agar

Ingredients	Gm/litre
Proteose peptone	10.000

Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Bile	8.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.000

1.3 Tryptone soya broth

Ingredients	Gm/litre
Casein enzymic hydrolysate	17.000
Papaic digest of soyabean meal	3.000
Dextrose	2.500
Sodium chloride	100.000
Dipotassium phosphate	2.500
Sodium pyruvate	10.000

1.5 MacConkey Broth

Ingredients	Gm/litre
Gelatin peptone	20.000
Lactose monohydrate	10.000
Dehydrated bile	5.000
Bromo cresol purple	0.010

1.6 Escherichia coli Broth (EC)

Ingredients	Gm/litre
Tryptone	20.000
Lactose	5.000
Bile salts mixture	1.500
Dipotassium hydrogen phosphate	4.000
Potassium dihydrogen phosphate	1.500
Sodium chloride	5.000

1.7 Phosphate buffered saline

Ingredients	Gm/litre
Sodium chloride	8.500
Disodium hydrogen phosphate	1.910
Potassium dihydrogen phosphate	0.380
Final pH (at 25°C)	7.2±0.2

1.8 Nutrient Agar

Ingredients	Gm/litre
Peptone	5.000
Sodium chloride	5.000
HM peptone B	1.500
Yeast extract	1.500
Agar	15.000

2. Biochemical characterization

2.1 Mannitol Salt Agar (MSA)

Ingredients	Gm/litre
Proteose peptone	10.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000

2.2 Tryptone broth (Indole medium)

Ingredients	Gm/litre
Tryptone	10.000
Sodium chloride	5.000

2.3 MR-VP Medium (Glucose Phosphate Broth)

Ingredients	Gm/litre
Peptic digest of animal tissue	5.000
Dextrose	5.000
Dipotassium phosphate	5.000

2.4 Simmon's citrate agar

Ingredients	Gm/litre
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000

Bromothymol blue	0.080
Agar	15.000

2.4 Nitrate Broth

Ingredients	**Gm/litre**
Peptic digest of animal tissue	5.000
Meat extract	3.000
Potassium nitrate	1.000
Sodium chloride	30.000

2.5 Hugh Leifson Medium

Ingredients	**Gm/litre**
Peptone	2.000
Sodium chloride	5.000
Dipotassium phosphate	0.300
Glucose (Dextrose)	10.000
Bromothymol blue	0.030
Agar	3.000

3. Antimicrobial susceptibility test

3.1 Mueller- Hinton Agar

Ingredients	**Gm/litre**
Beef infusion	300.000
Acicase	17.500
Starch	1.500
Agar	17.000

3.2 Antibiotic disc as per CLSI Standards

4. Molecular characterisation of ARGs

4.1 Polymerase Chain Reaction

All the reagents used were procured from ThermoFisher Scientific Ltd., USA and primers were procured from Bioinnovations Ltd., Mumbai.

4.2 Agarose Gel Electrophoresis

4.2.1 TAE Buffer (Tris-Acetate-EDTA) (50X)

Tris base-242g

Acetic Acid-57.1ml

0.5M EDTA-100ml

ddH_20-1 Litre

4.2.2 Ethidium bromide, 10 mg/ml

Ethidium bromide-0.2g

ddH_20-20ml

4.2.3 Gel loading buffer (6X)

Bromophenol blue- 0.25g

Xylene cyanol-0.25g

Glycerol-3ml

Made up the volume to 10ml using distilled water and stored at 4^0C

5. Staining Solutions

5.1 Crystal violet for Gram's stain

Solution A

Crystal violet - 2g

Ethyl alcohol - 20ml

Solution B

Ammonium oxalate - 0.8g

DW - 80ml

Mix solutions A&B, filter; keep overnight before use

5.2 Gram's iodine

KI - 2g

Iodine crystals - 1g

DW - 300ml

5.3 Safranine

Safranine 1g

Ethyl alcohol 40ml

DW 360ml

6. Indicator solution

6.1 Methyl Red Indicator

Methyl red 50 mg

Alcohol 150 ml

DW 100 ml

7. Test reagents

7.1 Kovac's cytochrome oxidase reagents

N:N:N:N Tetramethyl-p-phenylene diamine hydrochloride – 100mg

DW - 10ml

7.2 Kovac's indole reagent

P-dimethylamino benzaldehyde 5.000

Amyl alcohol 75.000

Hydrochloric acid, concentrated 25.000 -

7.3 VP test reagent

Solution A

a-Naphthol 0.25 gm

Absolute ethanol 5 ml

Solution B

Potassium hydroxide 2gm

Distilled water 5 ml

APPENDIX-III

QUESTIONNAIRE USED FOR THE SURVEY

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	Name of the Farm		
2	Owner Information		
3	Address		
4	Email		
5	Ph.no		

(B) FARM DETAILS

1	Total area of the farm			
2	GPS data	Latitude		
		longitude		
3	Ownership Style			Owned/leased/Friends& Relatives
4	Level of Management			Extensive/Intensive/Semi Intensive
5	Types of Pond			Fresh/Brackish water/Marine

(C) SAMPLING DETAILS

1	Species Cultured		
2	Days of Culture		
3	Average Length		
4	Average Weight		
5	Gut Content		
6	Feed type		
7	Probiotics used		
8	If yes, please specify		
9	Antibiotics used		

QUESTIONNAIRE- FARM 1

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Vikas Bari Farm
2	**Owner Information**		Mr Vikas Bari
3	**Address**		Dahanu, Dhumket, Thane, MAHARASHTRA, 401601
4	**Email**		-
5	**Ph.no**		9545726320

(B) FARM DETAILS

1	**Total area of the farm**			10 acres
2	**GPS data**	**Latitude**		19°56'52.0" N
		longitude		072°43'25.4" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		101
3	**Average Length**		15.2 cm
4	**Average Weight**		21.5 gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 2

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Harish Macchi Farm
2	**Owner Information**		Mr Harish Macchi
3	**Address**		Dahanu, Dhumket, Thane, MAHARASHTRA, 401601
4	**Email**		-
5	**Ph.no**		7798279589

(B) FARM DETAILS

1	**Total area of the farm**			1.6 acres
2	**GPS data**	**Latitude**		19°57'12.660" N
		longitude		072°43'26.880" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		110
3	**Average Length**		15.3 cms
4	**Average Weight**		20.4 gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 3

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Jaganath Hareshwar Macchi Farm
2	**Owner Information**		Mr Jaganath Hareshwar Macchi
3	**Address**		Dahanu, Chandigaon, Thane, MAHARASHTRA, 401103
4	**Email**		**-**
5	**Ph.no**		9226517991

(B) FARM DETAILS

1	**Total area of the farm**			5 acres
2	**GPS data**	**Latitude**		19°55'46.560" N
		longitude		072°43'46.560" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		Penaeus vannamei
2	**Days of Culture**		102
3	**Average Length**		15.2 cms
4	**Average Weight**		21.3gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S- Pellet
7	**Probiotics used**		No
8	**If yes, please specify**		-
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 4

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Ganesh Macchi
2	**Owner Information**		Mr Ganesh Macchi
3	**Address**		Dahanu, Agwan, Thane, MAHARASHTRA-401602
4	**Email**		-
5	**Ph.no**		9226267814

(B) FARM DETAILS

1	**Total area of the farm**			5 acres
2	**GPS data**	**Latitude**		19°56'36.960" N
		longitude		072°46'28.260" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		108
3	**Average Length**		15.6cms
4	**Average Weight**		22.8gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 5

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Pandurang Macchi Farm
2	**Owner Information**		Mr Pandurang Macchi Farm
3	**Address**		Dahanu, Agwan, Thane, MAHARASHTRA-401602
4	**Email**		-
5	**Ph.no**		9226173016

(B) FARM DETAILS

1	**Total area of the farm**			5 acres
2	**GPS data**	**Latitude**		19°55'59.700" N
		longitude		072°45'45.060" E
3	**Ownership Style**			leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		102
3	**Average Length**		15.8cms
4	**Average Weight**		20.5gms
5	**Gut Content**		Full
6	**Feed type**		Avanthi 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant-Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 6

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Pancham Aquaculture Farm
2	**Owner Information**		Mr. Ajit Patil
3	**Address**		Palghar, Saphale, Thane, MAHARASHTRA-401102
4	**Email**		-
5	**Ph.no**		9320508601

(B) FARM DETAILS

1	**Total area of the farm**			14 acres
2	**GPS data**	**Latitude**		19°31'40.980" N
		longitude		72°48'20.400" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		105
3	**Average Length**		14.2cms
4	**Average Weight**		20.1gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		No
8	**If yes, please specify**		-
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 7

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Shakti Aquaculture
2	**Owner Information**		Mr. Kirtiraj Keshav Salian
3	**Address**		Palghar, Saphale, Thane, MAHARASHTRA-401102
4	**Email**		**-**
5	**Ph.no**		9881943637

(B) FARM DETAILS

1	**Total area of the farm**			10 acres
2	**GPS data**	**Latitude**		19°32'15.5" N
		longitude		72°45'30.0" E
3	**Ownership Style**			Owned
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		109
3	**Average Length**		13.8cms
4	**Average Weight**		21.2gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		No
8	**If yes, please specify**		-
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 8

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Sajan Ashok Bari
2	**Owner Information**		Mr. Sajan Ashok Bari
3	**Address**		Dahanu, Bade Pokharan, Thane, MAHARASHTRA-401601
4	**Email**		-
5	**Ph.no**		9545726320

(B) FARM DETAILS

1	**Total area of the farm**			7 acres
2	**GPS data**	**Latitude**		19°57'34.560" N
		longitude		72°42'0.5400" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		Penaeus vannamei
2	**Days of Culture**		112
3	**Average Length**		16.1cms
4	**Average Weight**		24.2gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 9

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Rama Zepru Macchi
2	**Owner Information**		Mr. Rama Zepru Macchi
3	**Address**		Dahanu, Sawata, Thane, MAHARASHTRA-401602
4	**Email**		-
5	**Ph.no**		9226169337

(B) FARM DETAILS

1	**Total area of the farm**			6 acres
2	**GPS data**	**Latitude**		19°57'34.6" N
		longitude		72°41'59.5" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		109
3	**Average Length**		15.4cms
4	**Average Weight**		22.5gms
5	**Gut Content**		Full
6	**Feed type**		Avanti-3S Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 10

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Nilesh. Ramchandra. Mhatre
2	**Owner Information**		Mr. Nilesh. Ramchandra. Mhatre
3	**Address**		Dahanu, Bordi, Thane, MAHARASHTRA-401701
4	**Email**		-
5	**Ph.no**		9923284851

(B) FARM DETAILS

1	**Total area of the farm**			12 acres
2	**GPS data**	**Latitude**		20°06'52.740" N
		longitude		72°44'24.000" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		105
3	**Average Length**		14.6cms
4	**Average Weight**		20.5gmg
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant- Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 11

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Devendra Vasudev Patil Farm
2	**Owner Information**		Mr. Devendra Vasudev Patil
3	**Address**		Vasai, Chandrapada, Thane, MAHARASHTRA-401208
4	**Email**		-
5	**Ph.no**		9822797071

(B) FARM DETAILS

1	**Total area of the farm**			6 acres
2	**GPS data**	**Latitude**		19°22'38.9" N
		longitude		072°51'16.0" E
3	**Ownership Style**			Owned
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		102
3	**Average Length**		15.6cms
4	**Average Weight**		21.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 12

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Bhoir Farm
2	**Owner Information**		Mr Prakash Balaram Bhoir
3	**Address**		Palghar, Jalsar, Thane, MAHARASHTRA-
4	**Email**		-
5	**Ph.no**		9850367188

(B) FARM DETAILS

1	**Total area of the farm**			4 acres
2	**GPS data**	**Latitude**		19°31'48.4" N
		longitude		72°48'22.6" E
3	**Ownership Style**			Owned
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		101
3	**Average Length**		14.6cms
4	**Average Weight**		19.8gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 13

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		K.K.Aqua Farm
2	**Owner Information**		Mrs Vaishali Dhanmeher
3	**Address**		Palghar, Kharekuran, Thane, MAHARASHTRA-401405
4	**Email**		-
5	**Ph.no**		7720810374

(B) FARM DETAILS

1	**Total area of the farm**			8 acres
2	**GPS data**	**Latitude**		19°33'10.560" N
		longitude		72°47'8.4600" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		110
3	**Average Length**		16.8cms
4	**Average Weight**		24.8gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 14

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Bassein Aqua
2	**Owner Information**		Mr Yashwant Koli
3	**Address**		Vasai, Malaji Pada, Thane, MAHARASHTRA-
4	**Email**		-
5	**Ph.no**		8624841510

(B) FARM DETAILS

1	**Total area of the farm**			12 acres
2	**GPS data**	**Latitude**		19°21'33.7" N
		longitude		072°49'18.6" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		106
3	**Average Length**		15.6cms
4	**Average Weight**		23.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 15

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		MadhuBhai Farm
2	**Owner Information**		Mr.MadhuBhai.LeelaBhen. Patel
3	**Address**		Olpad, Kundiyana, Surat, GUJARAT- 394540
4	**Email**		-
5	**Ph.no**		9879199299

(B) FARM DETAILS

1	**Total area of the farm**			4 acres
2	**GPS data**	**Latitude**		21°19'9.9000" N
		longitude		72°39'33.780" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		106
3	**Average Length**		16.2cms
4	**Average Weight**		24.3gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 16

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		ShaileshKumar Farm
2	**Owner Information**		Mr.ShaileshKumar.RameshB hai. Patel
3	**Address**		Olpad, Kundiyana, Surat, GUJARAT- 394540
4	**Email**		-
5	**Ph.no**		8624841512

(B) FARM DETAILS

1	**Total area of the farm**			5 acres
2	**GPS data**	**Latitude**		21°19'53.280" N
		longitude		72°39'34.560" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		101
3	**Average Length**		16.1cms
4	**Average Weight**		20.9gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 17

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		MaheshBhai Farm
2	**Owner Information**		Mr.MaheshBhai.JamuBhai. Patel
3	**Address**		Olpad ,Saras, Surat, GUJARAT-394540
4	**Email**		-
5	**Ph.no**		9879545130

(B) FARM DETAILS

1	**Total area of the farm**			4acres
2	**GPS data**	**Latitude**		21°19'41.880" N
		longitude		72°39'30.960" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		106
3	**Average Length**		14.6cms
4	**Average Weight**		19.5gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 18

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		HarishBhai Farm
2	**Owner Information**		Mr.HarishBhai.MaganBhai Patel
3	**Address**		Olpad, Kapasi Surat, GUJARAT-394540
4	**Email**		-
5	**Ph.no**		9879209254

(B) FARM DETAILS

1	**Total area of the farm**			7 acres
2	**GPS data**	**Latitude**		21°20'27.840" N
		longitude		72°42'56.880" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		98
3	**Average Length**		13.6cms
4	**Average Weight**		18.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 19

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		SirishBhai Farm
2	**Owner Information**		Mr.SirishBhai.NanuBhai Patel
3	**Address**		Olpad,Kapasi, Surat, GUJARAT-394540
4	**Email**		-
5	**Ph.no**		9909663387

(B) FARM DETAILS

1	**Total area of the farm**			8 acres
2	**GPS data**	**Latitude**		21°20'26.280" N
		longitude		72°40'4.0200" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		102
3	**Average Length**		13.8cms
4	**Average Weight**		19.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 20

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Roy Farm
2	**Owner Information**		Mr.DurlaBhai.GovindBhai.Roy
3	**Address**		Olpad, Mor. Surat, GUJARAT-394530
4	**Email**		-
5	**Ph.no**		9427882121

(B) FARM DETAILS

1	**Total area of the farm**			10 acres
2	**GPS data**	**Latitude**		21°23'6.2400" N
		longitude		72°39'49.920" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		112
3	**Average Length**		15.3cms
4	**Average Weight**		22.5gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 21

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		MansukBhai Farm
2	**Owner Information**		Mr.MansukBhai.GovindBhai. Patel
3	**Address**		Olpad, Mor, Surat, GUJARAT-394530
4	**Email**		-
5	**Ph.no**		9427519534

(B) FARM DETAILS

1	**Total area of the farm**			3.5 acres
2	**GPS data**	**Latitude**		21°23'12.360" N
		longitude		72°39'43.140" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		97
3	**Average Length**		13.6cms
4	**Average Weight**		18.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 22

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		BaluBhai Farm
2	**Owner Information**		Mr.BaluBhai. Patel
3	**Address**		Olpad, Mor, Surat, GUJARAT-394530
4	**Email**		-
5	**Ph.no**		9427519534

(B) FARM DETAILS

1	**Total area of the farm**			6 acres
2	**GPS data**	**Latitude**		21°21'52.680" N
		longitude		72°40'7.0200" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		102
3	**Average Length**		15.9cms
4	**Average Weight**		23.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 23

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		JayeshBhai Farm
2	**Owner Information**		Mr.JayeshBhai.PravinBhai. Patel
3	**Address**		Olpad, Mor, Surat, GUJARAT-394530
4	**Email**		-
5	**Ph.no**		9979579629

(B) FARM DETAILS

1	**Total area of the farm**			6 acres
2	**GPS data**	**Latitude**		21°23'17.640" N
		longitude		72°40'2.8800" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		102
3	**Average Length**		15.1cms
4	**Average Weight**		22.4gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 24

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Iqbal Sheikh Farm
2	**Owner Information**		Mr Iqbal Sheikh
3	**Address**		Olpad, Delasa, Surat, GUJARAT-394530
4	**Email**		-
5	**Ph.no**		

(B) FARM DETAILS

1	**Total area of the farm**			8 acres
2	**GPS data**	**Latitude**		21°21'35.0" N
		longitude		72°40'48.7" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		100
3	**Average Length**		16cms
4	**Average Weight**		20.5gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 25

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Anup Patel Farm
2	**Owner Information**		Anup Patel
3	**Address**		Olpad, Sarsana, Surat, GUJARAT-395007
4	**Email**		-
5	**Ph.no**		9898285945

(B) FARM DETAILS

1	**Total area of the farm**			5 acres
2	**GPS data**	**Latitude**		21°04'41.7" N
		longitude		072°47'20.3" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		96
3	**Average Length**		13cms
4	**Average Weight**		18.1gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

QUESTIONNAIRE- FARM 26

As a part of my dissertation work of M.F.Sc thesis work, I am conducting, this survey. This questionnaire is intended to collect data for research and will be used for official purposes only.

(A) FARMER DETAILS

1	**Name of the Farm**		Umang Chopra Farm
2	**Owner Information**		Mr Umang Chopra
3	**Address**		Olpad, Sarsana. Surat, GUJARAT-395007
4	**Email**		-
5	**Ph.no**		9898285945

(B) FARM DETAILS

1	**Total area of the farm**			8acres
2	**GPS data**	**Latitude**		21°04'55.3" N
		longitude		72°47'40.1" E
3	**Ownership Style**			Leased
4	**Level of Management**			Semi Intensive
5	**Types of Pond**			Brackish water

(C) SAMPLING DETAILS

1	**Species Cultured**		*Penaeus vannamei*
2	**Days of Culture**		98
3	**Average Length**		15.1cms
4	**Average Weight**		20.3gms
5	**Gut Content**		Full
6	**Feed type**		Avanti 3S-Pellet
7	**Probiotics used**		Yes
8	**If yes, please specify**		Avant -Pro
9	**Antibiotics used**		Nil

PLATES

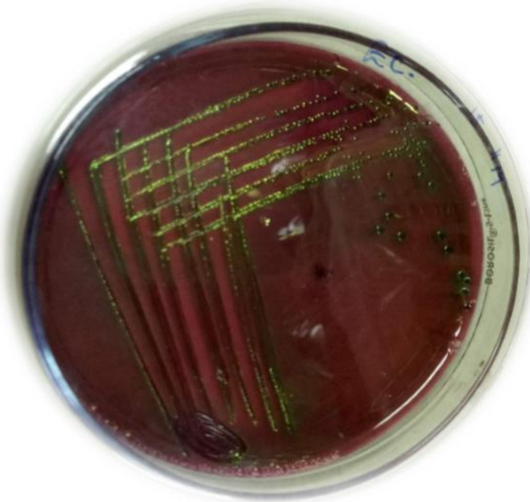


Plate 1: L-EMB agar plate with dark centered and flat, with green metallic sheen colonies.

Plate 2: BP agar with black shiny colonies with light coloured (off white) margin surrounded by an opaque zone.

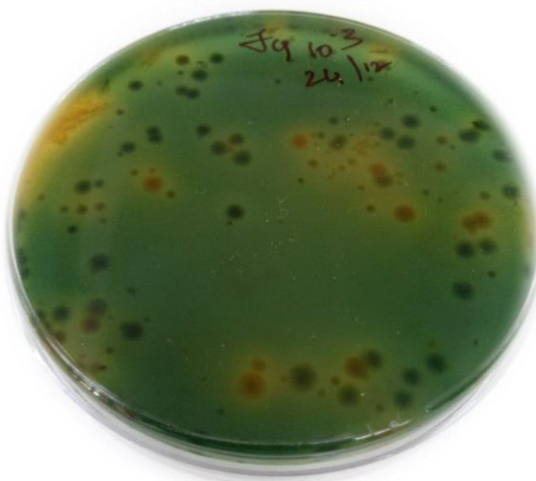


Plate 3: Green/Bluish-green centered, round, opaque colonies on TCBS agar

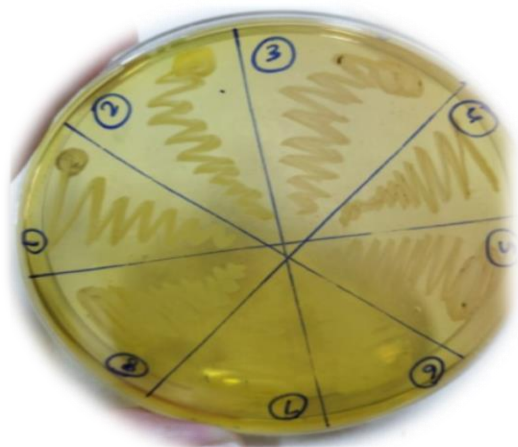


Plate 4: Pure cultures on nutrient agar

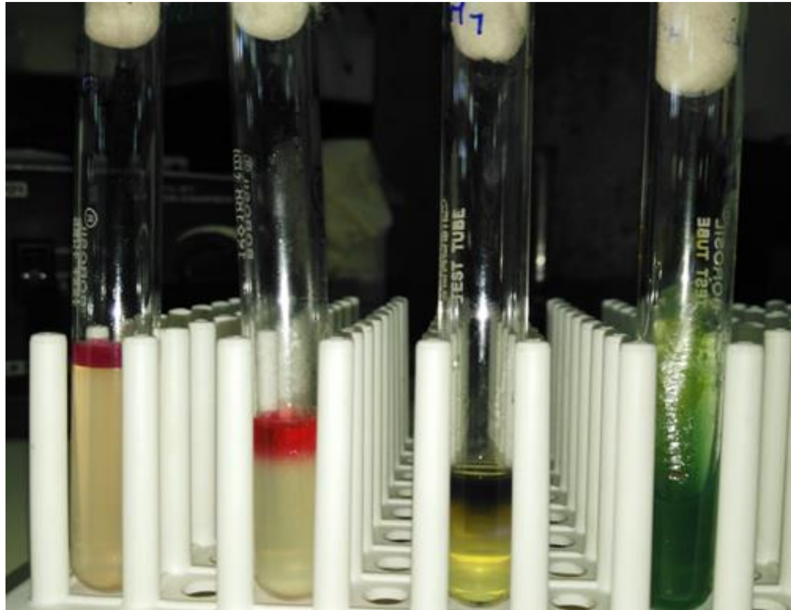


Plate 5: IMViC test results- (++--)

Plate 6: Yellow bright colonies of *S. aureus* on MS agar

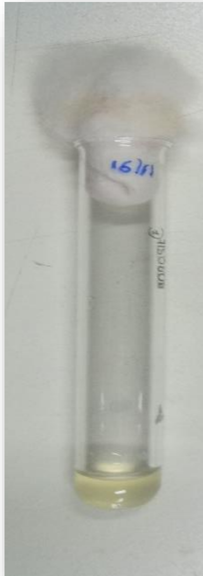
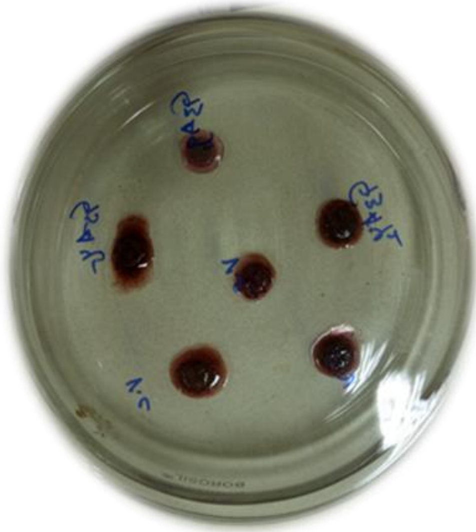


Plate 7: Coagulation of *S. aureus* culture



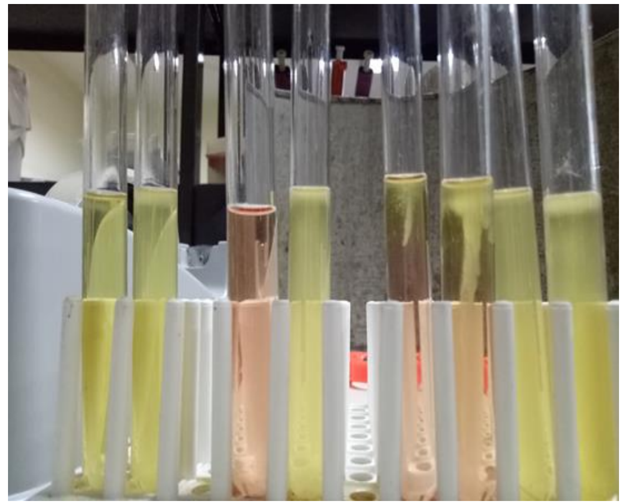


Plate 8: Nitrate reduction test result

Plate 9: Hugh and Leifson's oxidative / fermentative metabolism test result

Plate 10: VITEK 2 Compact system

Plate 11: MHA plates with impregnated antibiotics with zones of inhibition