Integrated Molecular and Phytochemical Approach for the Control of Multidrug-Resistant Bacteria in Fish Samples

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Abstract **- The emergence of multidrug-resistant (MDR) bacteria in aquatic environments poses serious risks to public health and food safety. This study aimed to isolate and characterize MDR bacteria from fish samples using microbiological and molecular techniques and to evaluate the antimicrobial potential of plant-derived extracts as an alternative remedial agent. Fish intestine and flesh samples were collected from a local market and processed under sterile conditions. Bacterial isolates were cultured and subjected to antibiotic susceptibility testing. Genomic DNA was extracted and analyzed using polymerase chain reaction (PCR) targeting the 16S rRNA gene, followed by agarose gel electrophoresis. Plant extracts of *Orthosiphon aristatus* were prepared using aqueous and ethanolic solvents, and rosemary oil was evaluated for antimicrobial activity using agar diffusion assays. The isolates exhibited resistance to conventional antibiotics, whereas plant extracts demonstrated significant inhibitory effects. The findings highlight the potential of phytochemicals as eco-friendly alternatives for controlling MDR bacteria in aquaculture.**

Keywords: **Multidrug resistance, Fish pathogens, DNA extraction, PCR, Plant extracts, Antimicrobial activity**

I. INTRODUCTION

Aquaculture is one of the fastest-growing food production sectors in India due to its crucial role in food security and nutrition [1]. Aquatic foods provide nearly 20% of animal protein and are a good source of vitamins, minerals (calcium, zinc), and omega-3 fatty acids [2].The increase in aquaculture production (from 13.1 million mt in 1990 to 82.1 million mt in 2018) has been attributed to the growing population (over 9 billion by 2050) and the increase in seafood consumption [3]. However, the specific nature of aquaculture practices such as the intensification, translocation, and introduction of aquaculture stocks, low genetic diversity, poor water quality, and the high stock densities, makes the organisms particularly susceptible to pathogen virulence and disease development [4]. It has been estimated that up to 50% of aquaculture production is lost due to diseases [5]. Aquatic ecosystems are reservoirs of diverse microbial populations, including pathogenic bacteria capable of affecting fish health and human consumers. The indiscriminate use of antibiotics in aquaculture and clinical settings has led to the rapid emergence of multidrug-resistant (MDR) strains, limiting therapeutic options and increasing environmental risks [6].

Fish-derived bacterial pathogens can act as carriers of resistant genes, facilitating their transfer through food chains. Conventional culture-based identification methods provide valuable information; however, molecular techniques such as polymerase chain reaction (PCR) offer enhanced specificity and sensitivity. DNA-based approaches enable accurate genetic characterization of microbial isolates [7].

In recent years, attention has shifted toward plant-derived antimicrobial compounds as sustainable alternatives to synthetic antibiotics. Medicinal plants contain bioactive metabolites with proven antibacterial properties. *Orthosiphon aristatus*, commonly known as Java tea, has been reported to possess therapeutic potential [11].

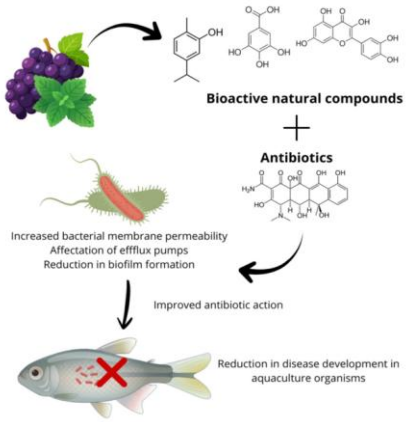


Fig.1: Disease management in aquaculture - efforts to limit antibiotic resistance.

Source: https://doi.org/10.3390/antibiotics15010095

Implementing effective surveillance systems to track antibiotic use and resistance patterns, along with public awareness initiatives promoting the consumption of responsibly sourced seafood, is crucial for fostering sustainable aquaculture practices. The World Health Organization and the Food and Agriculture Organization emphasize that global collaboration is vital to combat AMR in aquaculture, protect public health, and preserve aquatic ecosystems (FAO, 2021; WHO, 2020).

This study integrates microbiological, molecular, and phytochemical approaches to isolate MDR bacteria from fish samples, analyze their genetic material, and evaluate plant-based extracts for antimicrobial efficacy. The study reveals the importance of phytochemicals as alternative therapeutic agents in aquaculture and food safety management (9).

II. MATERIALS AND METHODS

A. Sample Collection

Fish samples were collected from a local market and transported to the laboratory in sterile containers. Intestine (FI) and flesh (FF) tissues were aseptically separated using forceps and dissection needle, and stored in test tubes for analysis.

B. Isolation and Cultivation of Bacteria

Samples were homogenized in distilled water using pestle and mortar and inoculated into nutrient agar and selective media plates. Plates were incubated at 37°C for 24 hours. Distinct colonies were sub-cultured repeatedly to obtain pure isolates.

C. Antibiotic Susceptibility Testing

Antibiotic sensitivity was determined using the disc diffusion method on Mueller–Hinton agar. Antibiotic discs were placed on inoculated plates and incubated. Zones of inhibition were measured and interpreted according to standard guidelines.

D. Genomic DNA Extraction

Bacterial cells were harvested by centrifugation. Pellets were re-suspended in the TE buffer and treated with lysozyme, followed by SDS-mediated lysis. Phenol–chloroform–isoamyl alcohol extraction was performed, and DNA was precipitated using isopropanol. Ethanol washing was conducted, and DNA was rehydrated in the TE buffer and stored at 4°C.

E. Polymerase Chain Reaction (PCR)

PCR amplification of the 16S rRNA gene was carried out in a 20 µL reaction mixture containing template DNA, forward and reverse primers, PCR master mix, and nuclease-free water. Thermal cycling was performed using standard denaturation, annealing, and extension steps.

F. Agarose Gel Electrophoresis

PCR products were analyzed on 1–1.5% agarose gels prepared in a 1× TAE buffer. Ethidium bromide was used for staining. DNA ladders served as molecular markers. Bands were visualized under UV illumination (13).

G. Preparation of Plant Extracts

Fresh leaves of *Orthosiphon aristatus* were washed, crushed, and extracted using distilled water and ethanol. Extracts were filtered and stored at 4°C. Rosemary oil was used as an additional phytochemical agent (8).

H. Antimicrobial Activity Assay

Agar well diffusion and disc diffusion methods were employed to assess antimicrobial activity. Wells were loaded with plant extracts and oil samples. Plates were incubated at 37°C, and zones of inhibition were recorded (12).

Zone of Inhibition observed in Levoflaxin in the samples of Fish flesh (FF) and Fish intestine (FI).

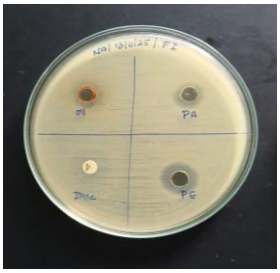
Sample	Antibiotic Disc	Zone of Inhibition (in mm)	Interpretation
FF	MET	Nil	Resistant
	AMP	Nil	Resistant
	P	Nil	Resistant
	LE	22	Sensitive
FI	MET	Nil	Resistant
	AMP	Nil	Resistant
	P	Nil	Resistant
	LE	22	Sensitive

MET, AMP, P and LE refers to the antibiotics Methicillin, Ampicillin, Penicillin and Levofloxacin Antibiotic susceptibility testing revealed resistance of bacterial isolates to commonly used antibiotics like Ampicillin, Penicillin and Methicillin. Inhibition Zone was observed in Levofloxacin.

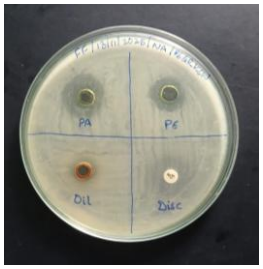
III. RESULTS

It can be observed that the given samples show resistance to antibiotics. As a result, plant extracts of *Orthosiphon aristatus* (Java tea) were used. This plant, also known as cat's whiskers or kidney tea, has many medicinal properties such as antimicrobial, antibiotic, diuretic, anti inflammatory and antioxidant properties, thus inhibiting the growth of the bacteria found in the fish samples. This can be noted from the below test which includes aqueous and ethanolic plant extracts of Java tea, Rosemary oil and the antibiotic disc Erythromycin.

Zone of Inhibition (mm)

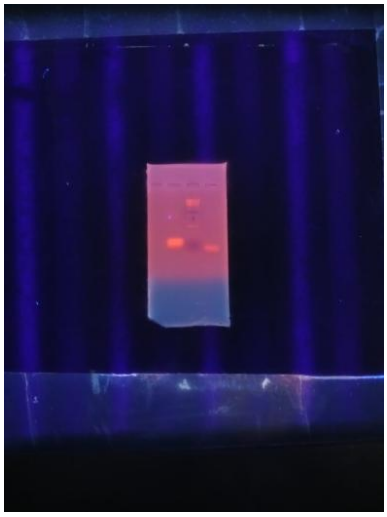


Zone of inhibition
Fish Intestinal (FI)
Plant extract



Zone of inhibition
Fish flesh (FF)
Plant extract

	Zone of Inhibition (in mm)			
Sample	PA (Aqueous Plant Extract)	PE (Ethanolic Plant Extract)	Rosemary Oil	Antibiotic Erythromycin
FI	12	14	11	Nil
FF	14	13	10	Nil



PCR amplification successfully generated DNA fragments corresponding to the 16S rRNA gene. Agarose gel electrophoresis confirmed the presence of amplified products. Subsequent identification of isolates will allow the correlation with antimicrobial resistance patterns, which can be further validated by detecting specific resistance genes.

IV. DISCUSSION

The presence of MDR bacteria in market fish samples reflects increasing antimicrobial resistance in aquatic environments. The resistance patterns observed may be attributed to excessive antibiotic usage and environmental contamination (9).

DNA extraction and PCR analysis enabled molecular confirmation of bacterial genetic material. The successful amplification of the 16S rRNA gene supports the reliability of the molecular protocol used.

Plant extracts demonstrated significant inhibitory effects against MDR isolates. Ethanolic extracts showed relatively higher activity, possibly due to enhanced solubility of bioactive compounds in organic solvents.
The integration of molecular and phytochemical approaches provides a comprehensive strategy for studying and controlling resistant bacteria in aquaculture systems (13, 14).

V. CONCLUSION

The study demonstrates that market fish can act as reservoirs of multidrug-resistant (MDR) bacteria, highlighting the growing concern of antimicrobial resistance in aquatic ecosystems. The successful use

of Polymerase Chain Reaction targeting the 16S rRNA Gene Sequencing confirms the effectiveness and reliability of molecular techniques for bacterial identification

Additionally, the observed antibacterial activity of plant extracts particularly ethanolic extracts suggests their potential as natural and sustainable alternatives to conventional antibiotics. The combined application of molecular identification and phytochemical screening offers a promising, integrated approach for monitoring and managing MDR bacteria in aquaculture.

Overall, this approach can contribute to reducing antibiotic dependence, mitigating resistance development, and promoting safer fish production and public health.

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