Springer Protocols

John Thomas
Natarajan Amaresan *Editors*

Aquaculture Microbiology

Humana Press

SPRINGER PROTOCOLS HANDBOOKS

For further volumes:
http://www.springer.com/series/8623

Springer Protocols Handbooks collects a diverse range of step-by-step laboratory methods and protocols from across the life and biomedical sciences. Each protocol is provided in the Springer Protocol format: readily-reproducible in a step-by-step fashion. Each protocol opens with an introductory overview, a list of the materials and reagents needed to complete the experiment, and is followed by a detailed procedure supported by a helpful notes section offering tips and tricks of the trade as well as troubleshooting advice. With a focus on large comprehensive protocol collections and an international authorship, *Springer Protocols Handbooks* are a valuable addition to the laboratory.

Aquaculture Microbiology

Edited by

John Thomas

Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Natarajan Amaresan

C.G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Surat, Gujarat, India

Humana Press

Editors
John Thomas
Centre for Nanobiotechnology
Vellore Institute of Technology
Vellore, Tamil Nadu, India

Natarajan Amaresan
C.G. Bhakta Institute of Biotechnology
Uka Tarsadia University
Surat, Gujarat, India

ISSN 1949-2448 ISSN 1949-2456 (electronic)
Springer Protocols Handbooks
ISBN 978-1-0716-3031-0 ISBN 978-1-0716-3032-7 (eBook)
https://doi.org/10.1007/978-1-0716-3032-7

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Preface

This manual consists of several chapters that deal with the techniques involved in the study of aquatic pathogens that cause infections, especially in fish. It covers a wide range of basic and advanced techniques associated with research on the isolation and identification of bacterial, viral, and fungal pathogens, and probiotic bacteria. In addition, it addresses the treatment of pathogens using seaweed extracts, medicinal plant extracts, and actinomycetes.

The knowledge and information shared in this manual provides information on the various protocols to be followed while performing the experiments. The editors are extremely grateful to each author or team of authors who found time to write a comprehensive chapter based on their expertise in various protocols. These protocols covered in this manual are widely followed by researchers and, therefore, will be extremely useful. Postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines in microbiology, biotechnology, and marine science are the targeted readers of this manual. Reading this manual will kindle further discussions among researchers working in aquaculture, biotechnology, microbiology, and other related subjects, thus widening its broader scope. Researchers will gain more knowledge of the information shared here on various protocols.

Vellore, Tamil Nadu, India — *John Thomas*
Surat, Gujarat, India — *Natarajan Amaresan*
October 2022

Contents

Part V Aquatic Actinomycetes and Copepods

Contributors

Raghu Ram Achar • *Division of Biochemistry, School of Life Sciences, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India*

Natarajan Amaresan • *C.G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Bardoli, Surat, Gujarat, India*

V. M. Amrutha • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

K. M. Anil Kumar • *Department of Environmental Science, JSS Academy of Higher Education & Research, Mysuru, India*

Ann Catherine Archer • *Department of Microbiology, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India*

R. Bharath • *VIT School of Agricultural Innovations and Advanced Learning (VAIAL), Vellore Institute of Technology, Vellore, Tamil Nadu, India; Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

N. Chandra Mohana • *Department of Microbiology, JSS Academy of Higher Education & Research, Mysuru, India*

Natrajan Chandrasekaran • *Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

Mirunalini Ganesan • *Centre for Ocean Research, Col. Dr. Jeppiaar Ocean Research Field Facility, Sathyabama Institute of Science and Technology (Deemed to be University), Chennai, Tamil Nadu, India*

Yamini Gopi • *Centre for Ocean Research, Col. Dr. Jeppiaar Ocean Research Field Facility, Sathyabama Institute of Science and Technology (Deemed to be University), Chennai, Tamil Nadu, India*

K. Karthikeyan • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

Ravi Mani • *Centre for Ocean Research, Col. Dr. Jeppiaar Ocean Research Field Facility, Sathyabama Institute of Science and Technology (Deemed to be University), Chennai, Tamil Nadu, India*

A. T. Manishkumar • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

Haimanti Mondal • *Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

Amitava Mukherjee • *Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

A. M. Nethravathi • *Post Graduate Department of Studies and Research in Biotechnology, Sahyadri Science College (Autonomous), Kuvempu University, Shivamogga, India*

Mahalakshmi S. Patil • *Department of Microbiology, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India*

Vernita Priya • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*

A. Saboor • *P.G. & Research Department of Zoology, The New College (Autonomous), Chennai, Tamil Nadu, India*

Sakthinarenderan Sai • *Centre for Ocean Research, Col. Dr. Jeppiaar Ocean Research Field Facility, Sathyabama Institute of Science and Technology (Deemed to be University), Chennai, Tamil Nadu, India*
S. R. Saranya • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*
Jalahalli M. Siddesha • *Division of Biochemistry, School of Life Sciences, JSS Academy of Higher Education & Research, Mysuru, India*
R. Sudhakaran • *Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*
Anagha Sudhama Jahgirdar • *Department of Studies in Zoology, University of Mysore, Mysuru, Karnataka, India*
S. Thanigaivel • *Department of Biotechnology, Faculty of Science & Humanities, SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu, India*
John Thomas • *Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India*
S. Vidhya Hindu • *Jaya College of Arts and Science, Thiruvallur, Tamil Nadu, India*
R. Vidya • *VIT School of Agricultural Innovations and Advanced Learning (VAIAL), Vellore Institute of Technology, Vellore, Tamil Nadu, India*
M. F. Yasmeen • *P.G. & Research Department of Zoology, Justice Basheer Ahmed Sayeed College for Women (Autonomous), Chennai, Tamil Nadu, India*

Part I

Isolation and Identification of Pathogens from Fishes

Chapter 1

Isolation and Identification of *Aeromonas* sp. from Fishes

Mirunalini Ganesan, Ravi Mani, and Sakthinarenderan Sai

Abstract

Aeromonas is an important bacterial pathogen that is frequently isolated from diseased fish throughout the world. Bacterial fish diseases, especially bacterial hemorrhagic septicemia and motile *Aeromonas* septicemia in freshwater fish, caused great losses. The researchers observed histopathological changes in intestines, the liver, and kidneys of the affected fish. Many researchers have isolated and identified *Aeromonas hydrophila* from carp, catfishes, perches, and eels and found that they are highly pathogenic. Here, we describe the methods used to isolate and identify *Aeromonas* spp. in fish by biochemical tests and other means. Our goals are to provide the reader with guidelines on how to identify *Aeromonas* infection and isolate the bacteria.

Key words Bacterial fish diseases, *Aeromonas*, Isolation, Identification, Characterization

1 Introduction

Aeromonas spp. are opportunistic pathogens that frequently cause infections as a result of host damage or stress [1]. Human illnesses caused by these bacteria include endocarditis, gastroenteritis, peritonitis, and septicemia [2]. They are also the most common infections in farmed fish [3]. *Aeromonas* spp., include *A. caviae*, *A. veronii*, *A. salmonicida* [4, 5], *A. hydrophila* [6], *A. sobria*, and *A. bestiarum*. Among these, *A. hydrophila* is thought to be the most dangerous to aquatic animals, producing hemorrhagic illness in farmed fish on a regular basis [7]. *A. veronii*, on the other hand, has recently been found to infect fish with many of the same symptoms and histological abnormalities as *A. hydrophila*. Virulence factors are used to measure virulence and toxicity of bacterial infections. The pathogenicity of *A. veronii* is mostly attributable to virulence factors and synergistic interactions. Virulence factors include cytotoxic enterotoxins (act, alt, ast), aerolysin (aer), polar flagella (fla), serine protease (ser), elastase (ahyB), lipase (lip), DNases (exu), glycerophospholipid: cholesterol acyltransferase (gcaT), and type III secretion system (ascV). To better understand

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_1,

the pathophysiology and epidemiology of *A. veronii*, it is critical to explore the virulence-related characteristics of clinical isolates [8]. However, data and information regarding sick fish are scarce.

To clarify what constitutes a favorable development platform for this pathogen, it is essential to analyze the growth properties of the bacterial isolate under various circumstances. *Aeromonas* is an aquatic-specific environmental bacterium that can be irregularly transferred to people [9, 10]. Foods derived from animals, fish, and vegetables have long been thought to be key carriers of *Aeromonas* spp. infections. Gastroenteritis is the most common human infection caused by *Aeromonas* spp.; however, other serious diseases, such as systemic infections, are less common and are usually associated with immunocompromised individuals. Furthermore, these microbes are known to cause major infections in fish. *Aeromonas* spp. can survive and multiply at low temperatures in a variety of food products stored between −2 and 10 °C, such as beef, roast beef, and pork, and can even produce virulence factors at these low temperatures. Although the incidence of foodborne outbreaks caused by *Aeromonas* spp. has been relatively low in the past, their presence in the food chain should not be overlooked. Little research has been conducted on the incidence of *Aeromonas* spp. in frozen fish, despite the fact that there have been many surveys on the prevalence of *Aeromonas* spp. in food products. Furthermore, these studies employed strains that were incorrectly identified using standard biochemical approaches, which resulted in unsatisfactory results.

2 Materials

2.1 Isolation of Aeromonas from Infected Fish

- Infected fish.
- Needles.
- Dissection kits.
- Petri plates.
- Incubator.

2.2 Staining and Biochemical Methods

- Refer to standard microbiology laboratory manual.

2.3 Molecular Identification

- PCR reaction fine chemicals as per standard protocols.
- Primers-F-5′ AGAGTTTGATCATGGCTCAG 3′,R-5′ GGTTACCTTGTTACGACTT3′.
- Thermocycler.
- Geldoc/transilluminator.

3 Methods

3.1 Fish Sampling

- Collect fish with clinical signs such darkening skin, external hemorrhages, and internal bleeding in the liver, kidneys, and skin, or a combination of these symptoms.
- Sterilize the equipment, such as needles, by spraying them with 70% alcohol and then expose them to the flames directly.
- Sterilize the Petri dishes by autoclaving them for 10–15 min at 121 °C and 1 atm pressure.
- Dissect liver, spleen, and kidneys from the fish after cleaning their skin with 75% ethyl alcohol.

3.2 Isolation of Bacteria

- Homogenize the dissected organs with 2 mL of PBS and centrifuge at 500 rpm for 5 min.
- Discard the supernatant and wash the pellet three times with PBS.
- Finally suspend the pellet with 1 mL of sterile PBS.
- Prepare Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar: pH 7) and autoclave it.
- Pour the autoclaved media into Petri plates and allow to solidify.
- Add 0.1 μL of the suspended pellet and spread plate it.
- Incubate it at 37 °C for 24–48 h and observe the colonies.

3.3 Identification of Aeromonas *Bacteria*

- *Aeromonas* is a rod-shaped, nonspore-forming, oxidase-positive, glucose-fermenting, facultatively anaerobic, and Gram-negative bacterium that lives in water.
- *Aeromonas* spp. colonies on trypticase soy agar are smooth, convex, and rounded, and they are tan/buff in color.
- Perform Gram staining as per the standard protocol.

3.4 Characterization of Aeromonas *Bacteria*

- *Aeromonas* spp. colonies on trypticase soy agar are smooth, convex, and rounded, and they are tan/buff in color. Characterization results of *Aeromonas* spp. are shown in Table 1.
- Weigh 25 g of fish flesh aseptically and homogenize for 2 min in stomacher bags containing 225 mL of alkaline peptone water.
- After 18 h of incubation at 37 °C, inoculate an aliquot of the enrichment in blood agar containing 30 mg/L ampicillin and incubate for 24 h at 28 °C.
- Select more or less than five presumptive *Aeromonas* colonies for further identification, but consider only one isolate representing a species from each sample for incidence calculation.

Table 1
The characterization result for the following *Aeromonas* spp. [12]

Characteristics	Results for the following species				
	A. hydrophila	***A. caviae***	***A. sobria***	***A. veronii***	***A. salmonicida***
Physiological characteristics					
Gram stain	–	–	–	–	–
Shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	Nd
Gas from glucose[a]	+	–	+	+	–
Methyl red	+	–	+	Nd	Nd
Voges–Proskauer[a]	+	–	–	+	–
Lysine decarboxylase[a]	+	–	+	+	Nd
Ornithine decarboxylase[a]	–	–	–	+	Nd
Vibriostatic 0/129	+	+	+	Nd	Nd
Production of:					
Indole[a]	+	+	+	+	+
Urease	–	–	–	Nd	–
Nitrate	+	+	+	Nd	–
Congo red	+	+	–	Nd	Nd
H2S from L-cysteine	+	–	–	–	Nd
Hydrolysis of:					
Arbutin[a]	+	+	Nd	Nd	Nd
Inulin	–	–	–	Nd	Nd
DNase	+	+	–	Nd	+
Elastin[a]	+	–	–	Nd	–
Esculin[a]	+	+	–	–	+
Starch[a]	+	+	+	–	Nd
Gelatin	+	+/–	–	Nd	Nd
Beta hemolysis	+	–	–	Nd	+
Alpha hemolysis	+	–	–	Nd	Nd
Prolin	+	+	+	Nd	Nd
Acid from:					

(continued)

Table 1
(continued)

Characteristics	Results for the following species				
	A. hydrophila	***A. caviae***	***A. sobria***	***A. veronii***	***A. salmonicida***
Adonitol	–	–	–	–	–
D-Arabitol	–	–	–	+	Nd
L-Arabitol	–	–	–	Nd	Nd
L- Arabinose[a]	+	–	–	Nd	+
D- Arabinose	+	+	–	Nd	Nd
D-Fucose	–	–	–	Nd	Nd
L-Fucose	–	–	–	Nd	Nd
Galactose	+	+	+	Nd	Nd
Gluconate	+	–	–	+	Nd
Dulcitol	–	–	–	–	Nd
Lactose	+	+	–	+	+
D- Mannitol[a]	+	+	+	Nd	+
Maltose	+	+	+	+	+
Melibiose	–	–	–	Nd	+
Inositol	–	–	–	–	–
D- Mannose	+	+	+	+	Nd
Salicin	+	+	–	–	+
Malonate	–	–	–	+	Nd
D- Sorbitol[a]	–	–	–	–	–
Saccharose (sucrose)[a]	+	+	+	+	+
Utilization of:					
Acetate	+	+	–	Nd	Nd
Arginine dihydrolase	+	+	–	–	Nd
Histidine	+	+	+	Nd	Nd
Lysine	+	+	+	Nd	Nd
Casein	+	–	–	Nd	Nd
Protease	+	+	–	Nd	Nd
Hemagglutination	+	–	–	Nd	Nd
Antibiotic susceptibility					
Ampicillin	R	R	R	R	R

(continued)

Table 1
(continued)

Characteristics	Results for the following species				
	A. hydrophila	*A. caviae*	*A. sobria*	*A. veronii*	*A. salmonicida*
Cephalothin	R	R	S	Nd	R
Gentamicin	R	R	R	S	NA
Penicillin	R	R	R	R	NA
Tetracycline	S	S	S	S	NA
Neomycin	S	S	S	M	NA
Carbenicillin	R	R	S	Nd	NA
Oxacillin	S	S	S	R	NA
Chloramphenicol	S	S	S	S	S
Nitrofurantoin	S	S	S	S	NA
Cefuroxime	S	S	S	Nd	NA
Cefotaxime	S	S	S	Nd	NA
Ciprofloxacin	R	R	R	S	NA
Clindamycin	R	R	R	R	NA
Erythromycin	R	R	R	M	R
Streptomycin	R	R	R	Nd	NA
Imipenem	S	S	S	S	NA
Kanamycin	R	R	R	S	NA
Piperacillin	S	S	S	R	NA
Polymyxin B	R	R	R	S	NA
Rifampicin	R	R	R	S	NA
Trimethoprim–sulfamethoxazole	S	S	S	Nd	NA

+ shows positive result, − shows negative result, +/− undeterminable, *Nd* not determined, *R* resistant against the antibiotic, *S* susceptible to antibiotic, *M* medium resistance, *NA* not available
[a]Major tests for *Aeromonas* spp.

- Maintain the stock cultures of each strain for short periods at room temperature on blood agar base slants and for longer storage, and either freeze at −70 °C in 20% (w/v) glycerol–Todd–Hewitt broth (Oxoid, Madrid, Spain) or lyophilize in 7.5% horse glucose serum as a cryoprotector.

3.4.1 Hemolytic Activity

- On an agar basis (Oxoid) supplemented with 5% sheep erythrocytes, the strains are evaluated for hemolytic activity.

- Streak each suspension over the plates with five microliters and incubate for 24 h at 22 °C and 37 °C.
- Presence of distinct colorless zone around the colonies indicates hemolytic activity [11].

3.4.2 Proteolytic Activity

- Determine casein hydrolysis by streaking each suspension onto Mueller–Hinton agar (Oxoid) containing 10% (w/v) skimmed milk (Difco, Barcelona, Spain) and incubate at 37 °C for 24 h.
- Caseinase activity is shown by the appearance of a translucent zone around the colonies. Each suspension is put in 4-mm-diameter wells cut into an agarose gel and incubate for 20 h at 22 °C.
- Precipitate unhydrolyzed gelatin by immersing the plates in a saturated ammonium sulfate solution at 70 °C.
- Gelatinase activity is shown by the appearance of a translucent zone around the colonies.

3.4.3 Lipolytic Activity

- Streak 5 mL suspension onto resazurin–butter agar and incubate at 37 °C for 24 h.
- Presence of pink colonies indicates lipase activity.

3.4.4 Nuclease Activity

- Extracellular nucleases (DNases) are determined on DNase agar plates (Difco) with 0.005% methyl green.
- Streak 5 µL bacterial suspension onto the plates and incubate at 37 °C for 24 h.
- A pink halo around the colonies indicates nuclease activity.

3.4.5 Congo Red Dye Uptake

- The ability to take up Congo red dye is determined on agar plates supplemented with 50 µg/mL/mL of Congo red dye.
- Streak 5 µL bacterial suspension onto the plates and incubate at 37 °C for 24 h.
- Orange colonies are considered positive and express different intensities in the dye uptake as + and ++.

3.4.6 Molecular Identification

Reidentify all strains on the basis of the restriction fragment length polymorphism patterns (RFLP) obtained from the 16S rDNA following the method described by Borrell et al. [9].

Add 200 µM each of dATP, dTTP, dCTP, and dGTP.

Add 5 µL 1× PCR buffer (50 mM KCL, 10 mM Tris–HCl, pH 8.3).

Add 5 µL 2.5 mM $MgCl_2$.

Add 2 μL of 5 pM of each primer.

Add 3 μL of 10 ng of genomic DNA.

Perform PCR using the following conditions: denaturation at 93 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. After the final cycle, extension at 72 °C is allowed for 10 min.

3.4.7 Antimicrobial Susceptibility

The resistance of all strains to different antimicrobial agents is determined by the disk diffusion method as described by Institute CALS [12].

Acknowledgments

The work is supported by the Ministry of Earth Sciences grant (MoES) (MoES/36/OOIS/53/2016), Govt. of India, and Sathyabama Institute of Science and Technology.

References

1. Jeney Z, Jeney G (2020) Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company' s public news and information
2. Janda JM, Abbott L (2006) The enterobacteria. ASM Press, Washington, D.C.
3. Hossain S, Dahanayake PS, De Silva BCJ et al (2019) Multidrug resistant Aeromonas spp. isolated from zebrafish (Danio rerio): antibiogram, antimicrobial resistance genes and class 1 integron gene cassettes. Lett Appl Microbiol 68:370–377. https://doi.org/10.1111/lam.13138
4. Zhu M, Wang XR, Li J et al (2016) Identification and virulence properties of Aeromonas veronii bv. sobria isolates causing an ulcerative syndrome of loach Misgurnus anguillicaudatus. J Fish Dis 39:777–781. https://doi.org/10.1111/jfd.12413
5. Long M, Nielsen TK, Leisner JJ et al (2016) Aeromonas salmonicida subsp. salmonicida strains isolated from Chinese freshwater fish contain a novel genomic island and possible regional-specific mobile genetic elements profiles. FEMS Microbiol Lett 363:1–8. https://doi.org/10.1093/femsle/fnw190
6. Chandrarathna HPSU, Nikapitiya C, Dananjaya SHS et al (2018) Outcome of co-infection with opportunistic and multidrug resistant Aeromonas hydrophila and A. veronii in zebrafish: identification, characterization, pathogenicity and immune responses. Fish Shellfish Immunol 80:573–581. https://doi.org/10.1016/j.fsi.2018.06.049
7. Marinho-Neto FA, Claudiano GS, Yunis-Aguinaga J et al (2019) Morphological, microbiological and ultrastructural aspects of sepsis by Aeromonas hydrophila in Piaractus mesopotamicus. PLoS One 14:1–20. https://doi.org/10.1371/journal.pone.0222626
8. Sen K, Rodgers M (2004) Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol 97:1077–1086. https://doi.org/10.1111/j.1365-2672.2004.02398.x
9. Borrell N, Acinas SG, Figueras MJ, Martínez-Murcia AJ (1997) Identification of Aeromonas clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J Clin Microbiol 35:1671–1674. https://doi.org/10.1128/jcm.35.7.1671-1674.1997
10. Janda JM, Abbott SL (2010) The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23:35–73. https://doi.org/10.1128/CMR.00039-09
11. Gerhardt P, Murray RGE, Castilow RN, Nester EW, Wood WA, Krieg NR, Phillips G (1981) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC
12. Institute CALS (2008) Pl Pl Pl. Quality 1–6

Chapter 2

Isolation and Identification of Edwardsiellosis-Causing Microorganism

Sakthinarenderan Sai, Ravi Mani, and Mirunalini Ganesan

Abstract

Isolation and identification of pathogenic bacteria are crucial for aquatic health management. Identifying the correct pathogenic bacteria at the species level is crucial in preparing for precautionary and disease management practices. Edwardsiellosis is an epidemic caused by *Edwardsiella* sp. This causes great economic losses for fish culture in marine and freshwater habitats. This chapter discusses various protocols for their isolation and identification.

Key words *Edwardsiella*, API 20E, Immunoassay blot, Edwardsiellosis polymerase amplification, PCR assay

1 Introduction

Edwardsiellosis is an important bacterial outbreak in most tropical aquaculture systems. The causative organism is Gram-negative rod bacteria belonging to the family *Enterobacteriaceae* and genus *Edwardsiella* [1]. It comprises five species: *E. tarda* [2], *E. ictaluri* [3], *E. hoshinae* [4], *E. piscicida* [5], and *E. anguillarum* [6]. The disease outbreaks have been reported for 20 species of freshwater and marine fish worldwide [7, 8]. The isolation and identification of pathogenic fish species are important for the control and prevention of major economic losses in aquaculture. This chapter describes the protocol for isolating and identifying different identification techniques used for *Edwardsiella* spp. from the infected fish species.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_2,

2 Materials

2.1 Isolation of Edwardsiella from Infected Fish

- Infected fish.
- Scalpel blades.
- Dissection kits.
- Petri plates.
- Incubator.

2.2 Staining and Biochemical Methods

- Refer to standard microbiology laboratory manual.

2.3 Molecular Identification

- PCR reaction fine chemicals as per standard protocols.
- Primers:
  1. EtfD- F 5′GGTAACCTGATTTGGCGTTC 3′.
     EttR 5′GGATCACCTGGATCTTAT CC 3′ [9].
  2. 16S rRNA- F 5′AGAGTTTGATCCTGGCTCAG 3′
     16S rRNA-R 5′ GGTTACCTTGT TACGACTT 3′ [10].
  3. EvpP- F 5′ GTGATCAAAGAAAACTGGAGCTCTCTCGACTT3′
     R EypP-R 5′GACCGTCAGGTTTGGAATATAGAA CTGTGT3′ [11].
- Thermocycler.
- Geldoc/transilluminator.

3 Methods

- Prepare bacterial isolates from organs such as the liver, intestine, and kidneys from the infested fish. Use sterile scalpel blades and take out samples aseptically.

3.1 Isolation of the Edwardsiella spp.

- After dissection, homogenize the tissue samples in physiological saline (0.5% NaCl solution).
- Take homogenate with sterile loop and streak on tryptic soy agar with 5% sheep blood or xylose lysine deoxycholate agar plate and then incubate it at 37 °C for 24 h.
- Round or circular or gray or grayish-white pigmentation of colonies after 24- or 48-h incubation is the characteristic feature of *Edwardsiella* spp.
- Examination of the bacterial isolates by phenotypic, biochemical, and molecular test to identify till species level.

3.1.1 Isolation of E. tarda, E. piscicida, *and* E. hoshinae *Species*

- Observed for small punctuate grayish-white colonies appearing on xylose lysine deoxycholate agar after 24 h of incubation at 37 °C.
- Subculture it on MacConkey agar plates and incubate at 37 °C for 24 h.
- Subculture all lactose non-fermented colonies on tryptic soy agar containing 0.5% NaCl and incubate it at 37 °C for 24 h.
- Perform all identification tests using overnight cultures in tryptic soy agar [12].

3.1.2 Isolation of Edwardsiella *spp.*

- Do the wet observation of infected fish (*E. ictaluri* is suspected).
- Streak the homogenate of tissue samples in trypticase soy agar with 5% sheep blood (SBA) or brain–heart infusion (BHI) agar plates and incubate at 26°C for 48 h.
- Subculture the isolate again in the same medium using 48-h incubated cultures.
- Perform all identification tests using overnight cultures in tryptic soy agar.

3.2 Preliminary Identification of Isolates

- Perform Gram staining and specific biochemical test for basic identification.

3.2.1 Gram Staining

- Ascetically, prepare a uniform smear of bacterial isolate in a clean glass slide using a drop of sterile water or saline solution.
- Allow it to air-dry and mildly fix it with heat.
- Then add few drops of crystal violet solution and left undisturbed for 1 min and wash using distilled water.
- Flood the smear with Gram's iodine for a minute.
- Then wash gently with distilled water or tap water until the violet color disappears.
- Decolorize using 95% ethyl alcohol until it runs clear and immediately rinse with water (5–10 s).
- After that add safranin (counterstain) for 45 s and again rinse with distilled water. Air-dry, blot-dry, and observe under optical microscope.

3.2.2 Biochemical Tests [12]

Catalase Test

- Detect catalase enzyme using hydrogen peroxide as substrate which results in water and oxygen.
- Perform this test using overnight-grown culture in TSA plates.
- *Edwardsiella* spp. shows catalase positive showing bubble formation in tubes or glass slide.

Oxidase Test

- Do this test to detect the presence of cytochrome oxidase enzyme in bacterial cell.
- Purple color formation confirms oxidase positive within 10 s on reaction with 1% aqueous solution of tetramethyl-phenylenediamine dihydrochloride.
- *Edwardsiella* spp. shows negative for oxidase test.

Triple Sugar Ion Test

- This test indicates the ability of bacteria to ferment lactose, sucrose, and glucose by producing H_2S gas production.
- *Edwardsiella* spp. shows positive for this test.

Indole Production Test

- Add Kovac's reagent to SIM (sulfur, indole, and motility) media.
- Incubate for 24 h and observe for the deep red coloration.
- *Edwardsiella* spp. shows positive for this test.

Simmons' Citrate Test

- Grow the bacterial isolate in Simmons' citrate slant by stabbing inoculum on the slant surface and incubate at 37°C for a week.
- Then observe for color change. *Edwardsiella* spp. shows negative result against this test indicating that it cannot use citrate as the only carbon source.

Lysine Decarboxylase Test

- Inoculate the bacterial isolate in lysine broth and incubate at 37°C for 4 days.
- Observe after 96 h of incubation. *Edwardsiella* spp. shows positive for this test.
- The purple color indicates the decarboxylation ability of isolate and maintain the alkaline pH of the medium.

3.2.3 Rapid Identification Using Commercial API20E Kit

- API20E is a plastic strip containing a set of 21 biochemical tests forming an identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods.
- Inoculate the bacterial isolate in microtubule substrate and incubate for identifying it.
- Give numerical values for the color changes of the substrate reaction (i.e., color changes), as per manufacturer guidelines.
- In total, seven-digit number is obtained for identifying species [13].

3.2.4 *Molecular Identification of Isolates*

- Isolate the DNA from bacterial strain either manually or using kit method.

DNA Isolation of *Edwardsiella* sp.

Pathogen Strain from Fishes

- A modified manual method using direct boiled cells method described by Ram Savan [14].
- Culture 1 mL of pathogenic isolates in the broth and centrifuge at 5000 × *g* for 3 min at 25 °C to obtain bacterial cell pellet.
- Boil the pellets at 95 °C for 5 min by resuspending it in 250 μL of TE buffer (10 mmoL/L Tris–HCl, 1 m mol/L Ethylenediamine tetraacetic acid (EDTA), pH 8.0).
- Again, centrifuge at same condition, and then collect supernatant and used it as template DNA.

DNA Extraction Directly from Spleen

- Dissect out a small part of spleen (20 mg) using sterile scalpel blade and aseptically transfer to 1.5 mL microcentrifuge tube.
- Extract the DNA using manual method or commercial kit.
- Use DNeasy Blood and Tissue kit from Qiagen and follow the manufacturer protocol for Gram-negative bacteria.
- Quantify the DNA extract using NanoDrop spectrophotometer.

Identification Using Conventional PCR Assay

- Prepare PCR mixtures by adding 1 μL of 16S rRNA/EvpP/etfD (forward; 10 μM), 1 μL EvpP/etfD (reverse; 10 μM) and 20 μL sterile distilled water.
- Set the PCR cycle to −95 °C for 5 min, and then 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 10 s at 72 °C and a final extension at 72 °C for 2 min.
- Determine the amplified products by 1% (w/v) agarose gel electrophoresis.
- Prepare the agarose gel in TAE (Tris–acetate–EDTA) buffer 1×, 0.04 m M Tris-HCL, 1 mM ethylenediaminetetraacetic acid, pH 8.0), and 0.06 μg/mL of ethidium bromide for visualization.
- Observed bands at the range from 400 to 445 base pairs.
- For species-level identification, purify the PCR products or elute using Qiaquick PCR cleanup kit (Qiagen) following the manufacturer protocol and sequence it through Illumina Next gen sequencer.
- Compare the sequences using BLASTN program from the National Center for Biotechnology Information [15].

Identification Using Recombinase Polymerase Amplification (RPA) Assay

RPA is a rapid, specific, and sensitive assay used for nucleic acid amplification by recombinase, single-chain binding protein and DNA polymerase under isothermal condition.

- Use 1 ng/μL concentration of DNA template and add to RPA reaction mixture.
- RPA reaction mixture consists of 0.42 μM RPA primers, 1× rehydration buffer, and DNase-free water (commercial kit: Cat. no. T00001, Jiangsu Qitian gene Biotechnology Co., China). To this mixture, add the DNA template.
- Leave the reaction undisturbed for 15 min at 39 °C and determine the amplified products confirmed by agarose gel electrophoresis method.
- For species-level identification, purify the PCR products or elute using Qiaquick PCR cleanup kit (Qiagen) following the manufacturer protocol and sequence using Illumina Next gen sequencer.
- Run the sequence using BLASTN program from the National Center for Biotechnology Information [11].

3.2.5 Identification of Edwardsiella *spp. Isolates by ELISA*

The whole cell protein profiling allows one to detect the strains very specifically using Immunoassay and ELISA.

Extraction of Whole Cell Protein (WCP)

- Inoculate overnight cultures of *Edwardsiella* spp. in tryptone soy broth (TSB) and incubate for 24 h at 37 °C.
- Fix the bacterial culture using 2% formalin and kept at 4 °C for 24 h. And then centrifuge at 2800 rpm for 45 min at 4 °C.
- Discard the supernatant and resuspend the pellet in phosphate buffer saline (pH 7.2). Again, centrifuge at 5000 rpm for about 15 min.
- After pelleting down, without disturbing them, keep it for sonication at 45 Hz for 10 min.
- After sonication, again centrifuge at 10,000 rpm for 15 min.
- The supernatant is collected and stored at −20 °C until further analysis. This supernatant is WCP antigen.

Production of Polyclonal Antibodies in Rabbit

- Inject the WCP antigen to a white rabbit on the hind leg to produce polyclonal antibodies.
- Inject 150 μg of WCP antigen along with the emulsion of Freund's complete adjuvant (FCA), intramuscularly.
- Give booster dose to the rabbit on 14th and 28th days of immunization.

- Collect the blood samples from rabbit on 42nd day post immunization and allow to clot at room temperature for 2–3 h.
- Centrifuge clotted blood samples at 5000 rpm for 10 min to obtain blood serum and store it at −20 °C.

SDS–PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) Analysis

- Mix the WCP antigen and 2× SDS gel loading buffer in 1:2 ratio and heat at 100 °C for 5 min.
- Do the electrophoresis of protein sample using 12% separating gel and 4% stacking gel.
- Allow the gel to run at 120 V until bromophenol blue dye reaches the bottom of the gel.
- Stain the gel with Coomassie brilliant blue R-250 followed by destaining.

Western Blotting of WCP Antigen

After SDS–PAGE, check the reactivity of the rabbit antiserum to the WCP antigen using Western blot immunoassay.

- Do the electrotransfer of running gel onto a nitrocellulose membrane (0.45 μm).
- Wash the membrane using distilled water, and block with 5% skimmed milk powder solution in phosphate buffer (PBS) containing 0.05% Tween 20 and incubate with primary antibody at 37 °C for 1 h.
- Again, wash it using PBS, and then incubate using secondary antibody (HRP conjugated goat anti-rabbit IgG) at 37 °C for 1 h.
- Finally wash using deionized water, and determine the bound antibodies by reacting it with 3,3′,5,5′-tetramethylbenzidine membrane peroxidase substrate.
- Stop the color reaction by rinsing to membrane with deionized water.

Dot-ELISA for Species Identification

- Coat the nitrocellulose paper strip with 3 μL of WCP antigen of *Edwardsiella* spp. and dry it for hours at room temperature.
- Then block the strip in PBS containing 5% skimmed milk powder at 37 °C for 30 min.
- Incubate for an hour at 37 °C, with primary antibody (1:400 dilutions).
- Again, wash the membrane strip for a few times and incubate with secondary antibody with same condition for 30 min.
- Further incubate the nitrocellulose paper with anti-bovine complex reagent for 30 min.

- Finally, incubate with 3-amino-9-ethylcarbazole (AEC) reagent for 10 min in dark.
- Wash the nitrocellulose membrane strip with distilled water, air-dry it at room temperature, and detect the blots [16].

Acknowledgments

The work is supported by the Ministry of Earth Sciences (MoES) MoES/36/OOIS/53/2016), Govt. of India, and Sathyabama Institute of Science and Technology.

References

1. Griffin MJ, Quiniou SM, Cody T, Tabuchi M, Ware C et al (2013) Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as E. tarda. Vet Microbiol 165:358–372
2. Ewing WH, McWhorter AC, LA Escobar MR (1965) *Edwardsiella*: a new genus of Enterobacteriaceae based on a new species, E. tarda. Int Bull Bacteriol Nomencl Taxon 15:33–38
3. Hawke JP (1979) A bacterium associated with disease of pond cultured channel catfish. J Fish Res Board Canada 36:1508–1512
4. Grimont PAD, Grimont F, Richard CSR (1980) *Edwardsiella hoshinae*, a new species of Enterobacteriaceae. Curr Microbiol 4:347–351
5. Abayneh T, Colquhoun DJSH (2013) *Edwardsiella piscicida sp.* nov.a novel species pathogenic to fish. J Appl Microbiol 114: 644–654
6. Shao S, Lai Q, Liu Q, Wu H, Xiao J et al (2015) Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813T encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as Edwardsiella anguillarum Sp. Syst Appl Microbiol 38: 36–47
7. Katharios P, Kokkari C, Dourala NSM (2015) First report of edwardsiellosis in cage-cultured sharp snout sea bream, Diplodus puntazzo from the Mediterranean: case report. BMC Vet Res 11:155
8. Yi G, Kaiyu W, Defang C, Fanling FYH (2010) Isolation and characterization of *Edwardsiella ictaluri* from cultured yellow catfish (Pelteobagrus fulvidraco). Isr J Aquac 62:105–115
9. Sakai T, Iida T, Osatomi KKK (2007) Detection of Type 1 fimbrial genes in fish pathogenic and non-pathogenic *Edwardsiella tarda* strains by PCR. Fish Pathol 42:115–117
10. Lan J, Zhang X-H, Wang Y, Chen J, Han Y (2008) Isolation of an unusual strain of *Edwardsiella tarda* from turbot and establish a PCR detection technique with the gyrB gene. J Appl Microbiol 105:644–651
11. Jiang J, Fan Y, Zhang S, Wang Q, Zhang Y, Liu Q, Shao S (2022) Rapid on-the-spot detection of *Edwardsiella piscicida* using recombinase polymerase amplification with lateral flow. Aquac Rep 22:100945
12. Kebede BHT (2016) Isolation and identification of *Edwardsiella tarda* from Lake Zeway and Langano, Southern Oromia, Ethiopia. Fish Aquac J 7:184
13. Natalija TP, Rozelindra C-R, Strunjak-Perovic I (2007) Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. Veterinárnímedicína 52:49–53
14. Savan R, Igarashi A, Matsuoka S, Sakai M (2004) Sensitive and rapid detection of edwardsiellosis in fish by a loop-mediated isothermal amplification method. Appl Environ Microbiol 70:621–624
15. Castro N, Toranzo AE, Núñez S et al (2010) Evaluation of four polymerase chain reaction primer pairs for the detection of *Edwardsiella tarda* in turbot. Dis Aquat Org 90:55–61. https://doi.org/10.3354/dao02203
16. Das BK, Sahu I, Kumari S et al (2014) Phenotyping and whole cell protein profiling of *Edwardsiella tarda* strains isolated from infected freshwater fishes. Int J Curr Microbiol App Sci 3:235–247

Chapter 3

Methods for Characterizing *Flavobacterium* in Fish

Yamini Gopi, Sakthinarenderan Sai, Mirunalini Ganesan, and Ravi Mani

Abstract

Bacterial gill disease, rainbow trout fry syndrome, Columnaris disease, and bacterial cold-water disease are caused by bacteria that are pathogenic to fish. They belong to the *Flavobacteriaceae* family. They are spotted with yellow pigmentation in aquatic organisms. These kinds of bacteria are isolated and identified using techniques such as Gram staining, motility, gliding motility, biochemical tests, and API 20E. It can also be identified using technical methods by plate count and microscopy. Molecular characterization such as DNA extraction, polymerase chain reaction (PCR), and phylogenetic analysis is also done to identify the species.

Key words *Flavobacteriaceae*, Yellow pigmentation, Gram staining, Motility, Gliding motility, Biochemical test, API 20E, Microscope, Plate count, DNA extraction, PCR, Phylogeny

1 Introduction

Flavobacterium spp. inhabiting fresh and marine environments may produce yellow-pigmented colonies on culture. It is very important to know the clinical information about bacterial cells to differentiate them into pathogens or saprophytes. Therefore, cells that produce yellow pigmentation, usually long and thin, adhere to the surface or epithelium of aquatic species belonging to the family *Flavobacteriaceae* [1]. The genus *Flavobacterium* comprises of more than 100 species [2]. Most of the *Flavobacterium* spp. are Gram-negative, long, slender gliding rods of 4–10 μm and 0.3–0.5 μm wide, strictly aerobic [3], and the colony sizes are round, convex, rhizoid, and flat, which grow an optimal temperature of 20–30 °C for most of the species. Among them, many are pathogenic and causative agents of various diseases in fish. *Flavobacterium psychrophilum* is the causative agent of rainbow trout fry syndrome and bacterial cold-water disease [4]. This type of disease occurs in cold water at 16 °C, with open lesions on the external surface of the fish. *Flavobacterium branchiophilum* and *Flavobacter-*

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_3,

ium succinicans are pathogens that cause bacterial gill diseases in fish [5]. *F. johnsoniae*, *F. hydatis*, *F. chilense*, *F. araucananum*, *F. oncorhynchi*, *F. plurextorum*, *F. tructae*, *F. piscis*, *F. collinsii*, *F. branchiarum*, *F. branchiicola*, and *F. spartansii* are other *Flavobacterium* spp. associated with fish diseases. Flavobacterial diseases are also pathogenic to amphibians and humans [6]. The isolation and identification of *Flavobacterium* spp. are based on morphological, biochemical, and molecular characterizations.

2 Materials

2.1 Isolation of Flavobacterium *spp.* from Fish

- Infected fish.
- Sterile swab.
- Shieh medium.
- Tobramycin.
- Cytophaga agar.
- Neomycin.
- Polymyxin B.
- Petri plates.
- Incubator.

2.2 Staining and Biochemical Test

- General staining, motility test, and biochemical were carried out as per standard protocols API20 E Kit.

2.3 Molecular Identification

- Shieh broth.
- Tobramycin.
- Incubator.
- Lyophilizer.
- Primers:
  27F:5′-AGAGTTTGATCMTGGCTCAG-3′.
  1387R:5′GGGCGGWGTGTACAAGGC-3′ [4].
  27F: 5′-AGAGTTTGATCMTGGCTCAG-3′.
  1492R:5′-TACGGYTACCTTGTTACGACTT-3′ [7].
  27F:5′- GAGTTTGATCMTGGCTCAG-3′.
  518R:5′WTTACCGCGGCTGG-3′ [7].
- Thermocycler.
- Gel doc.

3 Methods

3.1 Isolation of Flavobacterium spp. from Fish

- Collect the grayish/yellowish spot of bacterial samples from the head, gill, spleen, liver, and kidneys of fish with skin erosion, using a sterile swab.
- Streak immediately in Shieh medium supplemented with tobramycin [8] and Cytophaga agar (CA) supplemented with neomycin and polymyxin B to grow only selected bacteria with low nutrient requirement [9].
- The following media can be used for the isolation of *Flavobacteria*:

1. *Shieh medium* (peptone, 5 g; yeast, 0.5 g; sodium, 0.01 g; $BaCl_2(H_2O)_2$, 0.1 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.3 g; $FeSO_4.7H_2O$, 0.001 g; $NaHCO_3$, 0.05 g; tobramycin, 0.5 μg; agar (Dico), 10 g; distilled water, 1000 mL)
   - Add all the chemicals (except tobramycin) to distilled water and adjust the pH to 7.2.
   - Autoclave for 15 min at 121 °C.
   - Cool down and add membrane-filtered solution of tobramycin.
   - Mix and pour to Petri dishes and store at 4 °C.
2. *Cytophaga agar* (cryptone, 5 g; yeast, 5 g; sodium, 2 g; beef extract, 2 g; agar, 9 g/mL; neomycin, 5 μg/mL; polymycin, 10 U/mL; distilled water, 1000 mL)
   - Add all the reagents and adjust the pH to 7.2–7.4.
   - Autoclave at 121 °C for 15 min.
   - Cool the media and pour to Petri dishes and store at 4 °C.
   - Incubate the plates at 25 °C for 48 and 72 h. Check for yellow pigmentation in the inoculated plates and perform Gram staining for primary identification.

4 Identification

4.1 Technical Count

4.1.1 Total Bacterial Count

- Inoculate the bacterial sample in 1000 μL sterile water and make the serial dilution up to 10^3.
- Spread evenly in the plate and incubate at 25 °C at room temperature.

- Count the bacterial colonies by using a felt pen.
- Do calculation by CFU/mL = N × dilution × plate dilution [10].

4.1.2 Microscopy

- Isolate the affected area and take sections of 4 μm thickness and stain them with hematoxylin and eosin (H&E) and Giemsa, and then view under light microscopy [11].

4.2 Gram Staining

- Perform Gram staining to differentiate bacterial species.
- Make a smear on the slide from the bacterial colony.
- Air-dry it and add crystal violet to the smear and keep at room temperature for 5 min.
- Rinse the slide under running tap water to remove excess crystal violet.
- Add Gram's iodine dye and keep at room temperature for 2 min and wash with tap water.
- To remove excess nonspecific crystal violet staining, add decolorizer over the smear for 30 s and wash with tap water.
- Finally add safranin to the smear for 1 min and wash with tap water and observe under microscope (magnification, 100×).
- Gram-positive bacteria retain crystal violet color due to thick layer of peptidoglycan, and Gram-negative bacteria will appear in pink or red color due to thin layer of peptidoglycan [12].

4.3 Motility Test

- Perform motility test using a wet mount slide.
- Grow the bacterial culture in nutrient broth.

1. *Nutrient broth* (nutrient agar, 14 g; distilled water, 1000 mL)
   - Add the nutrient agar to the distilled water and maintain the pH 6.8.
   - Autoclave it at 121 °C for 15 min.
   - Cool down it and store it in 4 °C.
   - After the 24-h growth of bacterial culture in nutrient broth, take 15–20 μL of bacterial culture on the slide.
   - Cover the culture with a coverslip.
   - View under phase contrast microscope (magnification, 40×) [1].

4.4 Gliding Motility Test

- Perform gliding motility test by hanging drop slide method.
- Take a drop of the 48-h bacterial culture from the nutrient broth and place it in a clean coverslip.
- Cover the coverslip with a clean depression slide, so that the concavity of the lens is placed down.
- Turn the depression slide and place under the microscope (magnification, 40×) [1].

4.5 Biochemical Test

1. *Catalase test* (glass slide, 30% hydrogen peroxide)
   - Take a clean glass slide and make a smear of bacterial culture on it.
   - Add 30% hydrogen peroxide onto the bacterial cell.
   - Indication of bubbles results in positive reaction [1].
2. *Flexirubin pigmentation test* (glass slide, 20% KOH)
   - Take a clean glass slide and make a smear of bacterial culture on it.
   - Add 20% KOH on it and observe the color immediately.
   - The positive result indicates in reddish-purple or brown color [1].
3. *Oxidase test* (1% tetramethyl-p-phenylenediamine, filter paper, light protecting bottle, distilled water)
   - Prepare 1% tetramethyl-p-phenylenediamine in distilled water and cover it with light protecting bottle and store it in 4 °C.
   - Take a filter paper and make a smear of bacterial culture on it and add oxidase reagent (tetramethyl-p-phenylenediamine) to the bacterial culture.
   - Observe the color within 10 s.
   - The positive results indicates in purple color [13]
4. *Congo red absorption* (100 mg Congo red, 100 mL distilled water)
   - Weigh 100 mg of Congo red and add to 100 mL distilled water.
   - Mix well and store it in room temperature.
   - Add a few drops of Congo red solution to colonies of bacterial culture which are growing in cytophaga agar (CA).
   - After 2 min rinse with water.
   - Observe the color.
   - The positive result indicates in red color.

5. *DNase* (19.5 g DNase test agar, 1000 mL distilled water)
   - Petri dishes.
   - 1% HCl.
   - Add 19.5 g of DNase test agar to 1000 mL distilled water.
   - Autoclave it for 15 min at 121 °C.
   - Mix well and pour it to the Petri dishes.
   - Streak a bacterial culture onto the plate and incubate it for 72 h at 24 °C.
   - To precipitate the DNA, the plate is flooded with 1% HCl.
   - Observe the reaction.
   - A positive result indicates in clear zone around the bacterial streak [1].
6. *Gelatinase test* (1 g peptone, 0.25 g yeast extract, 3.75 g gelatin, 1 g NaCl (0.4%), 3.75 g agar, 250 mL distilled water)
   - Add the entire reagent to the distilled water and autoclave for 15 min at 121 °C.
   - Pour it to Petri plates and store at 4 °C.
   - Inoculate the bacterial culture on the gelatin plate.
   - Incubate it for 48 h.
   - A positive result indicates a clear or cloudy zone around the bacterial growth [1].

4.6 API 20E Commercial Identification Kit

- The plastic strips have 20 microtubes containing dehydrated differential media.
- Fill the dirots with water.
- Take the bacterial culture of the colony and make it to 5 mL with sterile water and homogenize the culture.
- By using sterile transfer pipette, fill each microtube.
- Cover the three wells (CIT, VP, and GEL) with more bacterial suspension, until the top of the well.
- Fill the five wells (ADH, LDC, ODC, H2S, and URE) with mineral oil to prevent oxygen from entering.
- Cover it and incubate it for 18 to 24 h at 37 °C.
- Observe the color.
- Add 1 drop of TDA reagent to TDA well and observe the color.
- Add 1 drop of Kovac's reagent to IND well and observe the color.
- Add 1 drop of each VP 1 and VP 2 reagent to VP well and wait for 10 min and observe the color.

- Note down the results in chart paper and calculate the seven-digit profile number.
- Look into appropriate database to identify the species [9].

5 Molecular Identification

5.1 DNA Extraction

- Inoculate the bacterial culture in Shieh broth with tobramycin and incubate for 48 to 72 h at 25 °C.
- Take 1.5 mL of bacterial culture and centrifuge for 2 min (repeat the process until the pellet forms).
- Discard the supernatant.
- Wash the cells with 567 μL TE buffer (10 mM Tris–HCl, pH 8, 1 mm EDTA).
- Add 30 μL of 10% sodium dodecyl sulfate (SDS) and 3 μL of 20 mg/mL of proteinase K.
- Incubate at 37 °C for 1 h.
- After incubation add 100 μL of 5 M NaCl (mix thoroughly) and add 80 μL of hexadecyl trimethyl ammonium bromide (CTAB reagent).
- Vortex to mix and incubate for 10 min at 65 °C.
- Add equal volume of chloroform/isoamyl alcohol (24:1) v/v and vortex gently for 20 s.
- Centrifuge it for 5 min at 10,000 rpm.
- Transfer the aqueous, viscous supernatant to the new micro-centrifuge tube and add equal volume of phenol/chloroform/isoamyl alcohol (25:24:2) v/v and centrifuge it for 5 min at 10,000 rpm.
- Transfer the supernatant to a fresh tube and add 400 μL of ice-cold isopropanol and centrifuge it for 5 min at 6500 rpm.
- Use 70% ethanol for the final wash and air-dry it for 5 min at room temperature.
- Remove the supernatant and dry the pellet by using lyophilizer and store it or redissolve in 100 μL TE buffer and store at −20 °C [14].

5.2 Polymerase Chain Reaction (PCR)

- Prepare PCR mixture for 20 μL containing the following: 110 ng of DNA, 0.5 μL of 10 mM dNTPs, 0.6 μL of 1.25 mM $MgCl_2$, 2 μL of 10× buffer solution, 1 μL of each primer (10 pmol/μL), 5 U Taq polymerase, and Milli-Q water.
- Carry out amplification under the following condition: initial denaturation at 94 °C for 2 min followed by denaturation at

94 °C for 30 s, primary annealing at 56 °C for 30 s, extension at 72 °C for 1 min, and then the final extension at 72 °C for 5 min [15].

- Prepare 1.5% agarose gel to analyze the PCR amplicon of 16 s rRNA and add 5 μg/mL of ethidium bromide (EtBr) as staining dye for 1 h and visualized under UV transilluminator.
- Approximately band length will be 1500 bp.

5.3 Product Purification Before Sequencing

- Take a microfuge and add 25 μL of 95% ethanol and 1 μL of 3 M sodium acetate and adjust the pH to 4.6.
- Add the entire PCR products and place on ice for 10–20 min.
- Centrifuge at 13,000 rpm for 30 min at room temperature and discard the supernatant.
- To the tube, add 125 μL of 70% ethanol and centrifuge it for 5 min at 13,000 rpm.
- Discard the supernatant and dry the pellet.
- Send the dried product to a sequencing facility [10].

5.4 Sequencing

- Sanger sequencing method produces the sequence reads.

5.5 Phylogenetic Analyzing

- Use MEGA version 4.0 software to analyze the phylogenetic relationship.
- Export the nucleotide sequence of the bacteria and align it in BLAST.
- Then retrieve the aligned sequence using neighborhood joining in MEGA software.
- Identify the phylogeny of the species using p-distance [4].

Acknowledgments

The work is supported by the Ministry of Earth Sciences grant (MoES) (MoES/36/OOIS/53/2016), Govt. of India, and Sathyabama Institute of Science and Technology.

References

1. De Aquicultura C (2008) Isolation and characterization of *Flavobacterium columnare* (Bernardet et al. 2002) from four tropical fish species in Brazil. Braz J Biol 68(2):409–414
2. P. Team (2016) Isolation and characterisation of flavobacteria from wild and cultured freshwater fish species in hungary. Acta Vet Hung 64(1):13–25. https://doi.org/10.1556/004.2016.002
3. Declercq AM, Haesebrouck F, Van Den Broeck W, Bossier P, Decostere A (2013) Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Vet Res 44:1–17

4. Starliper CE (2011) Bacterial Coldwater disease of fishes caused by *Flavobacterium psychrophilum*. J Adv Res 2(2):97–108. https://doi.org/10.1016/j.jare.2010.04.001
5. Good C, Davidson J, Wiens GD, Welch TJ, Summerfelt S (2015) Short Communication *Flavobacterium branchiophilum* and *F. succinicans* associated with bacterial gill disease in rainbow trout *Oncorhynchus mykiss* (Walbaum) in water recirculation aquaculture systems. J Fish Dis 2011:409–413. https://doi.org/10.1111/jfd.12249
6. Loch TP, Faisal M (2019) Emerging flavobacterial infections in fish: a review. J Adv Res 6(3):283–300. https://doi.org/10.1016/j.jare.2014.10.009
7. Sung JY et al (2018) Utility of conventional culture and MALDI-TOF MS for identification of microbial communities in bronchoalveolar lavage fluid in comparison with the GS junior next generation sequencing system. Ann Lab Med 38(2):110–118
8. Decostere A, Haesebrouck F, Devriese LA (1997) Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (Flexibacter columnaris) from diseased fish. J Clin Microbiol 35(1):322–324. https://doi.org/10.1128/jcm.35.1.322-324.1997
9. Altun S, Diler Ö, Ekici S (2008) Isolation of *Flavobacterium columnare* from cultured rainbow trout (*Oncorhynchus mykiss*) fry in Turkey. Turkish J Fish Aquat Sci 169:165–169
10. Buller NB (2014) Bacteria and fungi from fish and other aquatic animals: a practical identification manual. CABI, Wallingford, Oxfordshire
11. Tien NT, Dung TT, Tuan NA, Crumlish M (2012) First identification of *Flavobacterium columnare* infection in farmed freshwater striped catfish *Pangasianodon hypophthalmus*. Dis Aquat Organ 100:83–88. https://doi.org/10.3354/dao02478
12. Becerra SC, Roy DC, Sanchez CJ, Christy RJ, Burmeister DM (2016) An optimized staining technique for the detection of Gram positive and Gram-negative bacteria within tissue. BMC Res Notes 9(1):1–10. https://doi.org/10.1186/s13104-016-1902-0
13. Al-Dhabaan FA (2019) Morphological, biochemical and molecular identification of petroleum hydrocarbons biodegradation bacteria isolated from oil polluted soil in Dhahran, Saud Arabia. Saudi J Biol Sci 26(6):1247–1252. https://doi.org/10.1016/j.sjbs.2018.05.029
14. Andreou LV (2013) Preparation of genomic DNA from bacteria. Methods Enzymol 529:143–151. https://doi.org/10.1016/B978-0-12-418687-3.00011-2
15. Sebastião FA, Pilarski F, Lemos MVF (2010) Isolation and molecular characterization of *Flavobacterium columnare* strains from fish in Brazil. Adv J Microbiol Res 2(August):22–29

Chapter 4

Isolation and Identification of *Citrobacter* Species

Sakthinarenderan Sai, Ravi Mani, and Mirunalini Ganesan

Abstract

Isolation and identification of pathogenic bacteria is a crucial part of aquatic health management. Identifying the correct pathogenic bacteria at the species level is key to preparing precautionary and disease management practices. The pathogenic effects of *Citrobacter* sp. include tail necrosis, hemorrhage, reddening of the body, and lesions in the intestine. They are facultative pathogens that infect wild and cultured fish, turtles, aquatic mammals, and humans. It is an important threat to the aquaculture industry, leading to the mass mortality of cultured fish, thereby resulting in economic loss. This chapter discusses various protocols for their isolation and identification.

Key words *Citrobacter*, API 20E, PCR assay, Facultative pathogen, *C. freundii*, *C. braakii*

1 Introduction

The genus *Citrobacter* belongs to the *Enterobacteriaceae* family and consists of approximately 13 species [1]. *Citrobacter* is a Gram-negative, anaerobic bacterium that causes severe damage to the intestine and hepatopancreas in cultured fish, thereby leading to mass mortality. They are opportunistic pathogens in various fish, turtles, and humans [2]. *C. freundii* and *C. braakii* are the most infectious agents among other species of genus *Citrobacter*, which causes a great economic loss for the aquaculture industry [3]. Sato et al. were the first to isolate and describe the pathogenicity of *C. freundii* in diseased fish (*Mola mola*) [4]. Identification of pathogenic strains infecting fish is important for the control and prevention of major economic losses in aquaculture. This chapter deals with the protocol for isolating and identifying the different identification techniques used for *Citrobacter* species from infected fish.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_4,

2 Materials

2.1 Isolation of Citrobacter from Infected Fish

- Infected fish.
- Needles.
- Dissection kits.
- Petri plates.
- Incubator.

2.2 Staining and Biochemical Methods

- Refer to standard microbiology laboratory manual.

2.3 Molecular Identification

- PCR reaction fine chemicals as per standard protocols.
- PCR primer for 16S rRNA, Forward primer: UFF2: 5-′-GTTGATCATGGCTCAG-3′ and Reverse primer: URF2: 5′-GGTTCACTTGTTACGACTT-3′.
- Thermocycler.
- Geldoc/transilluminator.

3 Methods

3.1 Fish Sampling

- Collect fish with clinical signs of lethargy and anorexia, hemorrhage and hyperemia in the mouth, darkened skin color, and bilateral exophthalmia.
- Sterilize the equipment, such as needles, by spraying them with 70% alcohol and then expose them to the flames directly.
- Sterilize the Petri dishes by autoclaving them for 10–15 min at 121 °C and 1 atm pressure.
- Dissect the liver, spleen, and kidneys from the fish after cleaning their skin with 75% ethyl alcohol [5].

3.2 Isolation of Citrobacter

- Homogenize the dissected organs with 2 mL of PBS and centrifuge at 500 rpm for 5 min.
- Discard the supernatant and wash the pellet three times with PBS.
- Finally, suspend the pellet with 1 mL of sterile PBS.

3.3 Identification of Citrobacter *Bacteria*

- Straight Gram-negative rods, occurring singly and in pairs.
- Usually, motile using a peritrichous flagella.
- Pure culture colonies are opaque, 2–4 mm in diameter, smooth, and slightly convex and have a grayish color with a bright surface on TSA plates.
- On blood agar plates, colonies of the isolated bacteria are pink-violet and about 4 mm in diameter.
- XLD agar colonies are in yellow with an intensive yellow canter and a surrounding zone of yellow precipitation.
- In addition, the bacteria are motile, O/F fermentative, cytochrome oxidase negative, and catalase positive.
- Based on phenotypic characteristics, the bacterium is identified as *Enterobacteriaceae*.
- API 20E (bioMérieux) rapid test kit and VITEK II (bioMérieux, France) further identified the bacteria as *Citrobacter braakii* with probabilities of 99.9% and 90%, respectively [3, 6].

3.4 Rapid Identification Using Commercial API20E Kit

- API20E is a rapid commercial identification kit forming an identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods.
- It is a set of 21 biochemical tests consisting of dehydrated substrate microtubule.
- After inoculation of bacterial suspension in microtubules, the color changes of the substrate reaction are noted and numerical value is given to the bacteria, as per the manufacturer guideline.
- Then using a seven-digit number, the species is identified [7].

4 Molecular Identification of *Citrobacter* sp.

4.1 DNA Isolation

Employ heat treatment method and commercial kits to isolate bacterial DNA. It is a simple, cost-effective, and efficient method for extraction of bacterial DNA.

1. Use overnight cultures of pathogenic bacteria for extraction of genomic DNA.
2. Inoculate the cultures in test tubes containing 1 mL of sterile distilled water and keep in boiling water bath for 10 min.
3. Centrifuge it at 1000 rpm for 5 min. Collect the supernatant without disturbing pellet.
4. PCR analysis is done for the collected supernatant.
5. Check the DNA concentration using spectrophotometer or nanodrop.

4.2 16S rRNA Sequencing

- Isolate DNA from bacterial plates or directly from the infected organs of the fish.
- Prepare PCR mixture for 50 μL consisting of 100 ng of genomic DNA, 1 U Taq DNA polymerase, 5 μL of 10 × PCR buffer, 1 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTPs, and 1 μL of 10 pmol of each primer.
- Set the PCR cycle as follows: initial denaturation for 2 min at 95 °C, denaturation for 30 s at 95 °C (35 cycles), annealing at 52°C for 45 s, and extension for 1.30 min at 72 °C, and final extension at 72 °C for 7 min.
- Visualize PCR product using agarose gel (1.8%) and ethidium bromide stain.
- Further sequencing of PCR product is done using Illumina sequencer or ABI 3730xl capillary sequencer and analyze using NCBI -BLAST for confirmation of the species [8].

5 Biochemical Characterization (Table 1)

Table 1
Biochemical characterization of different *Citrobacter* sp.

Characteristics	Results for the following species			
	C. braakii	*C. freundii*	*C. gillenii*	*C. werkmanii*
Physiological characteristics				
Gram stain	–	–	–	–
Shape	Rod	Rod	Rod	Rod
Motility	+	+	+	+
Biochemical characteristics				
Catalase	+	+	+	+
Oxidase	+	+	+	+
Glucose (Hugh and Leifson)	+	–	+	+
Citrate (Simmons)	+	–	+	Nd
H2S	+	–	–	+
KCN growth	+	–	+	+
Methyl red	–	–	–	+
Voges–Proskauer	+	+	+	Nd

(continued)

Table 1
(continued)

Characteristics	Results for the following species			
	C. braakii	***C. freundii***	***C. gillenii***	***C. werkmanii***
Nitrate reductase	Nd	Nd	Nd	Nd
Gelatinase	+	+	+	+
Urease	−	−	−	Nd
Lysine decarboxylase	+	+	+	Nd
Ornithine decarboxylase	+	+	−	Nd
Phenylalanine deaminase	+	−	−	−
ONPG	Nd	Nd	Nd	Nd
Indole	+	+	Nd	Nd
Esculin	−	−	−	Nd
Gluconate	+	+	−	Nd
Tartrate	+	−	−	Nd
Glucose (gas)	+	+	−	−
Acids from				
Adonitol	+	+/−	−	Nd
Sorbitol	+	−	−	Nd
Mannitol	+	−	−	Nd
Dulcitol	+	+	+	Nd
Arabitol	Nd	Nd	Nd	Nd
Glucose	−	−	−	−
Lactose	−	−	−	+
Sucrose	−	−	−	Nd
Cellobiose	+	−	−	Nd
Melibiose	+	+	−	Nd
Raffinose	−	−	−	Nd
Xylose	−	−	−	Nd
Antibiotic susceptibility [6]				
Ampicillin	R	R	R	R
Cephalothin	R	R	S	Nd
Gentamicin	R	R	R	S
Penicillin	R	R	R	R

(continued)

Table 1
(continued)

Characteristics	Results for the following species			
	C. braakii	*C. freundii*	*C. gillenii*	*C. werkmanii*
Tetracycline	S	S	S	S
Neomycin	S	S	S	M
Carbenicillin	R	R	S	Nd
Oxacillin	S	S	S	R
Chloramphenicol	S	S	S	S
Nitrofurantoin	S	S	S	S
Cefuroxime	S	S	S	Nd
Cefotaxime	S	S	S	Nd
Ciprofloxacin	R	R	R	S
Clindamycin	R	R	R	R
Erythromycin	R	R	R	M
Streptomycin	R	R	R	Nd
Imipenem	S	S	S	S
Kanamycin	R	R	R	S
Piperacillin	S	S	S	R
Polymyxin B	R	R	R	S
Rifampicin	R	R	R	S
Trimethoprim–sulfamethoxazole	S	S	S	Nd

+ shows positive result, − shows negative result, +/− undeterminable, *Nd* not determined, *R* resistant against the antibiotic, *S* susceptible to antibiotic, *M* medium resistance, *NA* not available

Acknowledgments

The work is supported by the Ministry of Earth Sciences grant (MoES) (MoES/36/OOIS/53/2016), Govt. of India, and Sathyabama Institute of Science and Technology.

References

1. Anderson MT, Mitchell LA, Zhao L, Mobley HLT (2018) Citrobacter freundii fitness during bloodstream infection. Sci Rep 8:11792
2. Sun H-Y, Cao X-H, Jiang Y-F, Ni L-Y, Mo Z-Q, Qin Q-w, Li Y-W, Dan X-M (2018) Outbreak of a novel disease associated with Citrobacter freundii infection in freshwater cultured stingray, Potamotrygon motoro. Aquaculture 492:35–39
3. Altun S, Duman M, Buyukekiz A, Ozyigit M, Karataş Steinum S, Turgay E (2013) Isolation of Citrobacter braakii from rainbow trout (Oncorhynchus mykiss). Isr J Aquac 65:915–922.

4. Sato N, Yamane N, Kawamura T (1982) Systemic Citrobacter freundii infection among sunfish Mola mola in Matsushima aquarium. Bull Jpn Soc Sci Fish 48:1551–1557
5. Altun S, Duman M, Buyukekiz AG et al (2013) Isolation of Citrobacter braakii from rainbow trout (Oncorhynchus mykiss). Department of Aquatic Animal Disease, Faculty of Veterinary Medicine, Department of Pathology, Faculty of Veterinary Medicine, Uludag Department of Fish Disease, Faculty of F
6. Thanigaivel S, Vijayakumar S, Gopinath S, Mukherjee A, Chandrasekaran N, Thomas J (2015) Invivo and Invitro antimicrobial activity of Azadirachta indica (Lin) against Citrobacter freundii isolated from naturally infected Tilapia (Oreochromis mossambicus). Aquaculture 437: 252–255. 631471
7. Topic Popovic N, Coz-Rakovac R, Strunjak-Perovic I (2007) Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. Veterinárnímedicína 52:49–53
8. Dashti AA, Jadaon MM, Abdulsamad AM, Dashti H (2009) Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. Kuwait Med J 2:117–122

Chapter 5

Isolation and Identification of *Infectious Salmon Anemia Virus* from Shrimp

Haimanti Mondal, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

Infectious salmon anemia virus (ISAV) is a segmented RNA virus, belonging to the family *Orthomyxoviridae*. This virion causes infectious salmon anemia (ISA) in Atlantic salmon, *Salmo salar*. It is an infectious waterborne viral disease caused by *Salmon isavirus*. They mostly affect fish farming in Chile, Canada, Scotland, and Norway, causing heavy losses in salmonid aquaculture. The virus has been reported to survive in seawater which is a major risk for any aquacultural farm industry. Transmission of the virus is mostly spread by contact with infected fishes or their secretions, or with people handling them. There is no effective vaccine or treatment currently for ISAV.

Key words ISAV, ISA, Isavirus, Segmented, Salmonid, Aquaculture

1 Introduction

ISAV is an enveloped ssRNA virus that is reported to be closely associated with influenza viruses [1]. Infectious salmon anemia is the etiological agent of ISAV. They are placed within the kingdom *Orthornavirae*, phylum *Negarnaviricota*, class *Insthoviricetes*, order *Articulavirales*, family *Orthomyxoviridae*, and genus *Isavirus*. The genome consists of eight -ve sense single-stranded RNA (ssRNA) segments, coding approximately ten proteins [2].

Aamelfot et al. [3] reported a study on the ISAV mucosal infections in Atlantic salmon. They showed the early replication in various mucosal surfaces including gills, pectoral fin, GI tract, and skin, which are the entry points for the virus. The treatment and prevention strategies have been only partially effective. Similarly, Gervais et al. [1] conducted a study on the response of host to ISAV using single-cell RNA sequencing in an Atlantic salmon cell line.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_5,

They revealed the potential interaction of host–virus at cellular level which can be explored for increasing the resistance of Atlantic salmon to ISAV.

2 Materials

- Shrimp cell line.
- Tissue samples—the hepatopancreas, gills, liver, and heart.
- Shrimp cell culture medium (SCCM) (22 amino acids, 6 vitamins, 4 sugars, cholesterol, fetal bovine serum (FBS), 3 antibiotics, phenol red, disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 6.8–7.2).
- Gentamicin (50 μg/mL).
- Eppendorf tubes.
- PCR tubes.
- TRIzol reagent.
- Chloroform.
- Isopropanol.
- 70% ethyl alcohol.
- DEPC water.
- Primer pairs (forward and reverse).
- cDNA synthesis kit.
- Random hexamers (Promega).
- Homogenizer.
- NanoDrop spectrophotometer.
- UV transilluminator.
- Centrifuge.
- Microscope.

3 Methods

3.1 Dissection of Various Organs from shrimps infected with Infectious Salmon Anemia Virus *[4]*

- Dissect organs like the liver, hepatopancreas, gills, and heart from individual shrimp using a scalpel.

3.2 Isolation of Infectious Salmon Anemia Virus *from Cell Lines*

3.2.1 Sample Preparation

- Homogenize each sample using a mortar and pestle.
- Centrifuge the homogenate at 2–5 °C at 2000–4000 rpm for 15 min.
- Collect the supernatant and treat with antibiotic gentamicin (50 μg/mL) for 4 h at 15 °C or overnight at 4–8 °C.

3.2.2 Inoculation on Cell lines

- Take shrimp cells for primary isolation.
- Grow them in SCCM medium supplemented with 10% fetal bovine serum (FBS) and 2% (v/v) L-glutamine (200 mM) in standard concentrations.
- Incubate at 20 °C.
- Inoculate 100 μL/well of 1:50 diluted tissue homogenate of the shrimp cell line monolayers in well plates.

3.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) [5]

3.3.1 Extraction of RNA

- Remove a piece of the hepatopancreas and heart from the shrimp using a sterile scalpel and homogenize with 200 μL of NTE buffer.
- Centrifuge the samples at 12,000 rpm for 10 min.
- Add 250 μL of tissue homogenate with 600 μL TRIzol reagent.
- Incubate for 5 min in 1.5 mL Eppendorf tube.
- Add 200 μL chloroform and vortex it properly.
- Keep it in ice for 15 min or at −20 °C for 5 min.
- Centrifuge at 12,000 rpm, at 4 °C for 20 min.
- Collect the aqueous phase carefully without touching the interphase.
- Add 400 μL ice-cold isopropanol to the aqueous phase.
- Centrifuge at 12,000 rpm, at 4 °C for 15 min.
- Discard the supernatant and wash the pellet with 200 μL of ice-cold 70% ethanol.
- Centrifuge at 12,000 rpm, at 4 °C for 10 min.
- Discard the supernatant.
- Suspend the pellet with 40 μL of DEPC-treated water and store at −20 °C.
- Measure the purity and concentration of extracted RNA using NanoDrop spectrophotometer.

3.3.2 cDNA Synthesis

- Take 4 μL of RNA from 25 μL of total volume.
- Add 2 μL of oligodt primer.

- Make up the total volume to 20 μL with molecular biology grade water.
- Denaturation at 25 °C for 5 min and annealing at 42 °C for 60 min, followed by extension at 70 °C for 5 min (one cycle).

3.3.3 RT-PCR Assay [6]

- Design the PCR primers, forward primer (FP) (5′-CTACACAG CAGGATGCAGATGT -3′) and reverse primer (RP) (5-′-CAGGATGCCGGAAGTCGAT-3′) for ISAV.
- Carry out the amplification with 40 cycles—denaturation at 94 °C for 30 s, annealing at 61 °C for 45 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min.
- Run the electrophoresis on 2% agarose gel.
- Visualize under UV transilluminator at 304 nm.

References

1. Gervais O, Barria A, Papadopoulou A, Gratacap RL, Hillestad B, Tinch AE, Martin SAM, Robledo D, Houston RD (2021) Exploring genetic resistance to infectious salmon anaemia virus in Atlantic salmon by genome-wide association and RNA sequencing. BMC Genomics 22: 345
2. Cárdenas M, Michelson S, Pérez DR, Montoya M, Toledo J, Vásquez-Martínez Y, Martin MC-S (2022) Infectious salmon anemia virus infectivity is determined by multiple segments with an important contribution from segment 5. Viruses 14:631
3. Aamelfot M, McBeath A, Christiansen DH, Matejusova I, Falk K (2015) Infectious salmon anaemia virus (ISAV) mucosal infection in Atlantic salmon. Vet Res 46:120
4. Olesen NJ, Cuenca A, Iburg TM, Vendramin N (2019) Diagnostic methods for the surveillance and confirmation of infection with HPR-deleted *Infectious Salmon Anemia Virus* (ISAV). European Union Reference Laboratory for Fish and Crustacean Diseases. National Institute of Aquatic Resources, Technical University of Denmark
5. Kibenge FSB, Gárate ON, Johnson G, Arriagada R, Kibenge MJT, Wadowska D (2001) Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Dis Aquat Org 45:9–18
6. Cunningham CO, Snow M (2000) Genetic analysis of *Infectious Salmon Anemia Virus* (ISAV) from Scotland. Dis Aquat Org 41(1):1–8

Chapter 6

Isolation and Identification of *Betanodavirus* from Shrimp

Haimanti Mondal, John Thomas, Natrajan Chandrasekaran, Amitava Mukherjee, and Natarajan Amaresan

Abstract

Nervous necrosis virus (NNV) or *Betanodavirus* is a positive, single-stranded RNA (ssRNA) virus. It belongs to the family *Nodaviridae*. It is an aquatic pathogen that has been reported as hazardous. They are responsible for causing the disease outbreak in a wide variety of freshwater and marine juvenile as well as larval shrimp around the world. This virion is deadly affecting approximately 120 species of fishes also across the globe. They are affected by several factors, like temperature, biological load or UV. They are widely distributed in a varied range of temperatures in the aquatic environment.

Key words *Betanodavirus*, *Nodaviridae*, Hazardous, Outbreak, Biological load

1 Introduction

Betanodavirus is a non-enveloped, positively ssRNA virus with an icosahedral capsid which ranges from 25 to 34 nm in diameter. It belongs to the family *Nodaviridae*. From the last decade, the fish culture has been hugely impacted by this virus [1]. This disease causes 100% mortality. It particularly affects the juvenile and larval stages of freshwater and marine fishes. The *Betanodavirus* infection was first reported in Australia in barramundi, *Lates calcarifer* [1].

Recently, [2] conducted a study on the imported diseased samples of pearl gentian grouper fry from Thailand. The PCR analysis showed that the infection gave positive results for NNV. Similarly, [3] reported a comparative study on the viability of the NNV virus in high- and low-salinity seawater at different temperatures in culture medium. They revealed that the NNV strains upon exposure to oxygen and UV in an aquarium accelerated the inactivated infective particles. They showed that with increase in the incubation temperature, there was decrease in survival of the virus.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_6,

2 Materials

- Tissue samples—the hepatopancreas, gills, liver, and intestine.
- Shrimp cell line.
- Shrimp cell culture medium (SCCM) (22 amino acids, 6 vitamins, 4 sugars, cholesterol, Fetal Bovine Serum (FBS), 3 antibiotics, phenol red, disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 6.8–7.2).
- Membrane filter (0.22 μm size).
- Eppendorf tubes.
- PCR tubes.
- Homogenizer.
- Sodium chloride (NaCl).
- Chloroform.
- Isopropanol.
- 70% ethyl alcohol.
- TRIzol reagent.
- DEPC water.
- Primer pairs (forward and reverse).
- RNA easy mini kit (Qiagen).
- cDNA synthesis kit.
- Random hexamers (Promega).
- NanoDrop spectrophotometer.
- UV transilluminator.
- Cooling centrifuge.

3 Methods

3.1 Dissection of Various Organs from Shrimp

- Dissect various organs like the liver, hepatopancreas, gills, and intestine from shrimp using a sterile scalpel.

3.2 Isolation of Betanodavirus *Using Shrimp Cell Lines [4–6]*

- Homogenize the sample using homogenizer in 5 mL of SCCM medium without FBS.
- Centrifuge the homogenized tissue at 10,000 rpm at 4 °C for 20 min.
- Filter the suspension through a 0.22 μm size membrane filter.
- Grow the shrimp cell line in SCCM medium supplemented with 5% FBS.

- Inoculate the shrimp cell line monolayer with 500 µL of the collected filtrate and incubate at 27 °C for 1 h.
- Perform the RNA extraction and RT-qPCR.

3.2.1 RNA Extraction

- Homogenize 20 mg of hepatopancreas and gills with NTE buffer.
- Centrifuge at 10,000 rpm for 5 min.
- Collect 150 µL of the supernatant and add 600 µL of TRIzol reagent and incubate for 5 min.
- Add 200 µL chloroform and vortex it.
- Keep it in ice for 15 min or at −20 °C for 5 min.
- Centrifuge at 12,000 rpm, at 4 °C for 20 min.
- Collect the aqueous phase carefully without touching the interphase.
- Add 400 µL ice-cold isopropanol to the aqueous phase.
- Centrifuge at 12,000 rpm, at 4 °C for 15 min.
- Discard the supernatant and wash the pellet with 200 µL of ice-cold 70% ethanol.
- Centrifuge at 12,000 rpm, at 4 °C for 10 min.
- Discard the supernatant.
- Suspend the pellet with 25 µL of DEPC-treated water and store at −20 °C.
- Measure the purity and concentration of extracted RNA using NanoDrop spectrophotometer.

3.2.2 cDNA Synthesis

- Take 4 µL of RNA from 25 µL of total volume.
- Add 2 µL of oligodt primer.
- Make up the total volume to 20 µL with molecular biology grade water.
- Denaturation at 25 °C for 5 min and annealing 42 °C for 60 min, followed by extension at 70 °C for 5 min.

3.3 RT-PCR [2, 7]

- Design the primer sets for genomic amplification of RNA.
- Use forward primer, F (5′-CTT-CCT-GCC-TGA-TCC-AAC-TG-3′), and reverse primer, R (5′-GTT-CTG-CTT-TCC-CAC-CAT-TTG-3′).
- Carry out the reaction in a total volume of 25 µL—each primer (200 nM), RNA template (500 ng), and 1 µL SuperScript III RT/Platinum Taq Mix (Invitrogen).

- Carry out the amplification for reverse transcription—pre-denaturation at 50 °C for 30 min and denaturation at 94 °C for 2 min, amplification with 30 cycles—denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 3 min/1.5 min, and final extension at 72 °C for 5 min.
- Run the RT-PCR amplicons on agarose gel electrophoresis.
- Visualize under UV transilluminator.

References

1. Shetty M, Maiti B, Santhosh KS, Venugopal MN, Karunasagar I (2012) Betanodavirus of marine and freshwater fish: distribution, genomic organization, diagnosis and control measures. Indian J Virol 23(2):114–123
2. Jungi SV, Sangsuriya P, Taengphu S, Phiwsaiya K, Sonthi M, Nuangsaeng B, Salin KR, Senapin S, Dong HT (2022) Detection of nervous necrosis virus RGNNV genotype in pearl gentian grouper *(Epinephelus lanceolatus* ♂ × *E. fuscoguttatus* ♀*)* fry imported to Thailand. Aquaculture 561:738659
3. Vázquez-Salgado L, Olveira JG, Bandín I (2022) Nervous necrosis virus viability modulation by water salinity and temperature. J Fish Dis 45(4):561–568
4. Yamashita Y, Fujita Y, Kawakami H, Nakai T (2005) The efficacy of inactivated virus vaccine against viral nervous necrosis (VNN). Fish Pathol 40:15–21
5. Hick P, Tweedie A, Whittington R (2010) Preparation of fish tissues for optimal detection of betanodavirus. Aquaculture 310:20–26
6. Chen X, Qi J, He L, Luo H, Lin J, Qiu F, Wang Q, Zheng L (2022) Isoaltion and identification of a new strain of nervous necrosis virus from the big-belly seahorse *Hippocampus abdominalis*. Virol J 19:109
7. Nishi S, Yamashita H, Kawato Y, Nakai T (2016) Cell culture isolation of Piscine Nodavirus (Betanodavirus) in fish-rearing seawater. Appl Environ Microbiol 82(8):2537–2544

Chapter 7

Isolation and Identification of *Hemorrhagic Septicemia Virus* from Shrimp

Haimanti Mondal, John Thomas, Natrajan Chandrasekaran, Amitava Mukherjee, and Natarajan Amaresan

Abstract

Viral hemorrhagic septicemia virus (VHSV) is an enveloped, −ve sense single-stranded RNA (ssRNA) virus. They belong to the family of rhabdoviruses that are responsible for enormous deaths of fishes worldwide. It causes *viral hemorrhagic septicemia* (VHS), a deadly pathogenic fish disease, afflicting more than 50 marine and freshwater fish species around the globe. The mortality of VHSV varies based on various physiological and environmental factors like stress, temperature, water, rearing condition, fish species, fish age, virus strain, and so on. Transmission of this infection primarily takes place horizontally through contaminated water by excreting the virus directly from infected fishes.

Key words Rhabdoviruses, VHSV, VHS, Rearing, Transmission, Pathogenic

1 Introduction

VHSV is a typical bullet or rod-shaped rhabdovirus consisting of 11–12 kb of RNA genome, encoding six proteins in the order 3′-Asn-Pro-Met-Gly-Val-Leu-5′ [1]. It belongs to the order *Mononegavirales* and are placed in the family *Rhabdoviridae*, and genus *Novirhabdovirus* [2]. They enter the host's cell through endocytosis or fusion. It binds to fibronectin, a glycoprotein through integrin receptor in the extracellular matrix. Originally, it was isolated in 1962 in Denmark from rainbow trout, *Oncorhynchus mykiss*.

Cieslak et al. [3] conducted a study based on the phylogeny of VHSV in the European aquaculture. They reported that the life of the most valuable aquacultural fish in Europe, rainbow trout, *Oncorhynchus mykiss*, was threatened by VHSV. Similarly, another study was conducted by Baillon et al. [1] on the VHSV markers in rainbow trout, *Oncorhynchus mykiss*. They displayed that these

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_7,

markers have great importance in predicting the in vivo phenotype of several viral isolates and also in improving prophylactic methods like developing live-attenuated safe vaccines.

2 Materials

- Hemolymph, tissue samples—the hepatopancreas, gills, and heart.
- Eppendorf tubes.
- PCR tubes.
- Shrimp cell culture medium (SCCM) (22 amino acids, 6 vitamins, 4 sugars, cholesterol, fetal bovine serum (FBS), 3 antibiotics, phenol red, disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 6.8–7.2).
- Shrimp cell line.
- Gentamicin (1 mg/mL).
- Sodium chloride (NaCl).
- Isopropanol.
- Chloroform.
- 70% ethyl alcohol.
- TRIzol reagent.
- DEPC water.
- Primer pairs (forward and reverse).
- RNA easy mini kit (Qiagen).
- cDNA synthesis kit.
- Random hexamers (Promega).
- Homogenizer.
- NanoDrop spectrophotometer.
- UV transilluminator.
- Centrifuge.

3 Methods

3.1 Dissection of Organs from Hemorrhagic Septicemia Virus *[4]*

- Dissect organs like the hepatopancreas, gills, and heart from shrimp using a sterile scalpel.

3.2 Isolation of Hemorrhagic Septicemia Virus Using Cell Line

- Homogenize the tissue samples completely using a homogenizer.
- Centrifuge at 2–5 °C at 2000–4000 rpm for 15 min.
- Collect the supernatant and treat with gentamicin (1 mg/mL) for 4 h at 15 °C or overnight at 4 °C.

3.3 Inoculation of the Cell Monolayers [5–7]

- Inoculate the supernatant onto confluent monolayers of shrimp cell line.
- Culture in shrimp cell culture medium (SCCM) with isosmotic seawater containing 27% salinity supplemented with streptomycin (100 mg/L), penicillin (100 IU/mL), chloramphenicol (0.06 mg/mL), and N-phenylthiourea (0.2 mM).
- Adjust the osmolarity to 720 ± 10 mOsm/kg.
- Incubate the inoculated monolayer at 25 °C for 7 days.

4 *Hemorrhagic Septicemia Virus* Identification

4.1 Real-Time Polymerase Chain Reaction (RT-PCR) Detection

4.1.1 RNA Extraction

- Homogenize 20 mg of hepatopancreas and gills with NTE buffer.
- Centrifuge at 10,000 rpm for 5 min.
- Collect 150 μL of the supernatant and add 600 μL of TRIzol reagent and incubate for 5 min.
- Add 200 μL chloroform and vortex it.
- Keep it in ice for 15 min or at −20 °C for 5 min.
- Centrifuge at 12,000 rpm, at 4 °C for 20 min.
- Collect the aqueous phase carefully without touching the interphase.
- Add 400 μL ice-cold isopropanol to the aqueous phase.
- Centrifuge at 12,000 rpm, at 4 °C for 15 min.
- Discard the supernatant and wash the pellet with 200 μL of ice-cold 70% ethanol.
- Centrifuge at 12,000 rpm, at 4 °C for 10 min.
- Discard the supernatant.
- Suspend the pellet with 25 μL of DEPC-treated water and store at −20 °C.
- Measure the purity and concentration of extracted RNA using NanoDrop spectrophotometer.

4.1.2 cDNA Synthesis [8]

- Take 4 μL of RNA from 25 μL of total volume.
- Add 2 μL of oligodt primer.
- Make up the total volume to 20 μL with molecular biology grade water.
- Denaturation at 25 °C for 5 min and annealing at 42 °C for 60 min, followed by extension at 70 °C for 5 min (one cycle).

4.1.3 RT-PCR [9, 10]

- Design the primer pairs, forward primer (FP) (5′-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3′) and reverse primer (RP) (5′-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3′) to carry out RT-PCR.
- Carry out the reaction in a total volume of 25 μL—each primer (200 nM), RNA template (500 ng), and 1 μL SuperScript III RT/Platinum Taq Mix (Invitrogen).
- Carry out the amplification with 40 cycles—denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s followed by final extension stage of 72 °C for 10 min.
- Run the RT-PCR amplicons on agarose gel electrophoresis.
- Visualize under UV transilluminator.

References

1. Baillon L, Mérour E, Cabon J, Louboutin L, Vigouroux E, Alencar ALF, Cuenca A, Blanchard Y, Olesen NJ, Panzarin V, Morin T, Brémont M, Biacchesi S (2020) The viral Haemorrhagic Septicaemia virus (VHSV) markers of virulence in rainbow trout (*Oncorhynchus mykiss*). Front Microbiol 11:574231
2. Dietzgen RG, Kondo H, Goodin MM, Kurath G, Vasilakis N (2017) The family *Rhabdoviridae*: mono-and bipartite negative-sense RNA viruses with diverse genome organization and common evolutionary origins. Virus Res 227:158–170
3. Cieslak M, Mikkelsen SS, Skall HF, Baud M, Diserens N, Engelsma MY, Haenen OLM, Mousakhani S, Panzarin V, Wahli T, Olesen NJ, Schütze H (2016) Phylogeny of the viral hemorrhagic septicemia virus in European aquaculture. PLoS One 11(10):e0164475
4. OIE (2019) Manual of diagnostic tests for aquatic animals. In: *Viral haemorrhagic septicaemia*. World Organisation for Animal Health (OIE), Paris
5. Jayesh P, Jose S, Philip R, Singh ISB (2013) A novel medium for the development of in vitro cell culture system from *Penaeus monodon*. Cytotechnology 65:307–322
6. Ahmadivand S, Soltani M, Mardani K, Shokrpoor S, Rahmati-Holasoo H, Mokhtari A, Hasanzadeh R (2016) Isolation and identification of viral hemorrhagic septicemia virus (VHSV) from farmed rainbow trout (*Oncorhynchus mykiss*) in Iran. Acta Trop 156:30–36
7. Zhang W, Li Z, Xiang Y, Jia P, Liu W, Yi M, Jia K (2019) Isolation and identification of viral haemorrhagic septicaemia virus (VHSV) isolate from wild largemouth bass *Micropterus salmoides* in China. J Fish Dis 00:1–10
8. Duesund H, Nylund S, Watanabe K, Ottem KF, Nylund A (2010) Characterization of a VHS virus genotype III isolated from rainbow trout (*Oncorhynchus mykiss*) at a marine site on the west coast of Norway. J Virol 7:19
9. Williams K, Blake S, Sweeney A, Singer JT, Nicholson BL (1999) Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J Clin Microbiol 37(12):4139–4141
10. OIE (2013) Manual of diagnostic tests for aquatic animals, 6th edn. World Organisation for Animal Health (OIE), Paris. http://www.oie.int/internationalstandard-setting/aquatic-manual/access-online/

Chapter 8

Isolation and Identification of Pathogens from Fish: Tilapia Lake Virus (TiLV)

S. R. Saranya, Vernita Priya, A. T. Manishkumar, and R. Sudhakaran

Abstract

Tilapia cultivation is extremely profitable and the industry in India is rapidly expanding. China is the world's largest producer of tilapia. Tilapines are disease-resistant fish compared to other fish, especially when it comes to many infections that target intensively farmed fish. They can still be infected with protozoan parasites and bacteria. Infectious and toxic etiologies should be considered in investigations of outbreaks of significant illness in fish. This protocol provides the details of tilapia lake virus, an emerging novel virus that causes mortality in tilapia species. The tilapine virus is becoming an epidemic that spreads through various routes in many countries. Therefore, it is necessary to gather knowledge about this deadly virus to prevent and treat the disease and minimize the economic loss of the country.

Key words Tilapia, Cultivation, Virus, World, Mortality

1 Introduction

The history of fish aquaculture began 3000 years ago with the simultaneous development of fish virology. Fish culture started in China with cyprinid fish, and many fish were cultured for food and ornamental trading, including Nile tilapia from Egyptian tombs. Viral infection of fish started as early as 1563, with spring viremia of carp and carp pox. Tilapia species were first identified in Africa and Middle East. A few decades ago, tilapia became an important species for aquaculture growth, with a worldwide harvest of eight million metric tons. Tilapia is now the second largest aqua species to be cultured after carp. It is a perennial breeder capable of tolerating water quality and pollution. Although there are different varieties of farmed tilapia, the most culturable species are Nile tilapia, Mozambique tilapia, and blue tilapia. Nile tilapia became famous as "aquatic chicken" by the National Fisheries Development Board in 2015 [1].

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_8,

Tilapia fishes are susceptible to different diseases, which can be considered a great economy loss for the country. Diseases occur upon infection by various pathogens such as viruses, bacteria, fungi, and protozoa. The common viral infections in tilapia fish include *Betanodavirus*, *Iridovirus*, and herpes-like viruses. Recently, the emergence of a novel viral pathogen called tilapia lake virus (TiLV) caused severe mortality in farmed fishes within 10 days of infection [2].

Tilapia tilapinevirus (TiLV) is the transboundary disease for *Tilapia* species which was first discovered in Israel followed by other countries, namely, Bangladesh, Chinese Taipei, Colombia, Egypt, Ecuador, India, Indonesia, Malaysia, Mexico, Peru, the Philippines, Thailand, etc., and also emerging to other countries.

1.1 Tilapia Lake Virus

Tilapia tilapinevirus (TiLV) is a negative-sense single-stranded RNA virus of *Amnoonviridae* family, genus Tilapinevirus, and *Tilapia tilapinevirus* species. The size of the virus is approximately 60–100 nm. TiLV has ten segments with 10,323 kb of total length and is enclosed within a membrane-bound nucleocapsid [3]. All segments contained an open reading frame (ORF), but segment 1 had weak sequence homology with the influenza C virus PB1 subunit (Bacharach et al. 2016). No other segments of TiLV have been found to be homologous to other known viruses [4]; however, the conserved complementary sequences were found to be consistent with the genome organization of orthomyxoviruses [3]. The life span of the virus inside the host lasts for 7–10 days. The host range for this virus is restricted to tilapine species (*Sarthoredon* and *Oreochromis* spp. and hybrids). The cohabitation of the infected fish with other species such as carps and mullets is not susceptible to this virus. However, recent studies have revealed that the virus can be transmitted to giant gouramis through cohabitation [5]. The viral particles of TiLV are sensitive to organic solvents such as ether and chloroform [4]. TiLV-infected tilapines are shown in Fig. 1.

Fig. 1 TiLV infection in tilapia fish [6]

2 Materials

2.1 Maintenance of Fish and Reinfection

- Fish.
- Fish tank.
- Aeration unit.
- Tilapia fishes.
- Rice husk powder.
- NTE buffer (50 mM Tris–HCl (pH 7.4); 100 mM NaCl; 0.1 mM EDTA).
- Syringe.
- Mortar and pestle.
- Eppendorf tubes.

2.2 RNA Extraction

- TRIzol or RNA isoplus.
- Chloroform.
- Isopropanol.
- Ethanol.
- Eppendorf tubes.
- Cooling centrifuge.
- RNAse-free water.
- NanoDrop.

2.3 cDNA and RT-PCR

- cDNA conversion kit.
- Thermocycler.
- Taq polymerase.
- Sterile water.
- dNTPs.
- Primers.
- Agarose.
- Electrophoresis tank.
- TBE buffer (90 mM Tris base; 90 mM boric acid; 2 mM EDTA).

2.4 Diagnosis of TiLV

- Primers.
- TiLV fish samples.
- Thermocycler.
- Taq DNA Master Mix.
- Gel electrophoresis.

- Gel documentation system.
- Real-time thermocycler.
- SYBR Green DNA Master Mix.
- ISH probe—DIG-labeled.

2.5 Morphological Examination of TiLV

- 10% formalin.
- Hematoxylin and eosin.
- Compound microscope.
- 2% glutaraldehyde.
- 1% osmium tetroxide
- Uranyl acetate and lead citrate.
- Transmission electron microscope.

3 Methods

3.1 Isolation of Tilapia tilapinevirus (TiLV)

1. Collect the tilapia fishes from the infected lake where the mass mortality witnessed or from the fish farms for reinfection through intraperitoneal injection.
2. Dissect out the infected fishes ($n = 5$) immediately and preserve in TRIzol at 4 °C for RNA extraction.
3. For reinfection, maintain fishes in the laboratory conditions for at least 5 days and provide feed twice a day to fishes.
4. Prepare required volume of positive TiLV homogenate from the infected fish and inject into healthy tilapia fishes intraperitoneally [7].
5. Monitor the injected fishes daily for the development of clinical symptoms at least for a period of 5–6 days.
6. Once the symptoms developed, collect the tissue from moribund stage fish and preserve in TRIzol.

3.2 Preparation of TiLV Homogenate

1. Prepare the homogenate by crushing the fish tissue in mortar and pestle with 1:10 (w/v) concentrate NTE buffer with 10 mM HEPES (pH 7.4–8.0).
2. Centrifuge the tissue homogenate at 12,000 rpm for 10 min at 4 °C and filter the supernatant through 0.22 μm filter.
3. Freeze-thaw the clear homogenate soup thrice to get bacterial breakdown.
4. Screen the final soup for TiLV positive by RT-PCR and use it for reinfection in healthy tilapia fishes.
5. Store the homogenate at −20 °C for further use.

3.3 RNA Extraction and RT-PCR

1. Dissect the different organs of TiLV infected fish like the gills, brain, liver, intestine, muscle, and kidney.
2. Weigh 50–100 mg of the infected fish tissue for total RNA extraction.
3. Follow the instruction of kit for RNA extraction.
4. Measure the purity and concentration of RNA using NanoDrop.
5. Use the isolated RNA to synthesize cDNA using cDNA conversion kit (reverse transcription) method.
6. Perform PCR with the synthesized cDNA as a template, the primers specific to TiLV virus, and Taq DNA polymerase.
7. Keep the cocktail in the thermal cycler with the following conditions: 95 °C for 30s, 56 °C for 30s, and 72 °C for 30s and final elongation at 72 °C for 10 min.
8. Repeat the above conditions for 30–32 cycles.
9. Once the PCR run completed, analyze the samples by agarose gel electrophoresis.
10. Visualize the results under Gel documentation system as shown in the diagrammatic representation in Fig. 2.

3.4 Diagnosis of TiLV

TiLV-infected tilapines are identified by different diagnosis methods like reverse transcriptase PCR, nested PCR and semi-nested PCR, quantitative real-time PCR [7, 8], RT-LAMP method, and in situ hybridization.

3.4.1 Nested PCR

1. To perform nested PCR, design two sets of primers for the successive PCR reaction.
2. Design the first pair of primer to anneal the sequence above the second pair of primers.

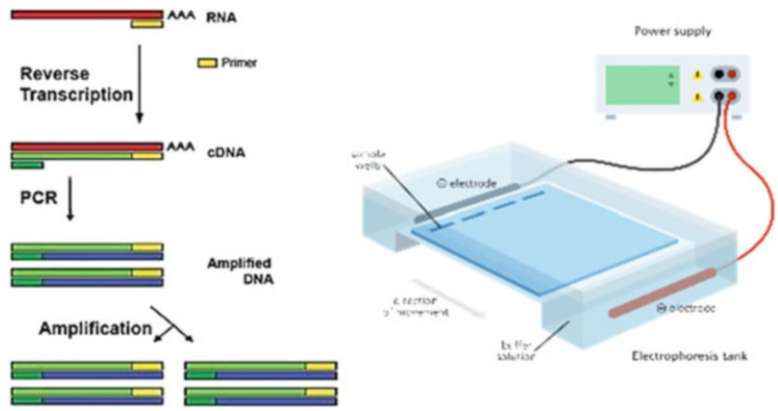

Fig. 2 Mechanism of reverse transcriptase PCR and gel electrophoresis

3. Use the reaction mixture resulting from the first PCR as a template for second PCR reaction which enhances the sensitivity and specificity of TiLV amplification [9].

3.4.2 Semi-nested PCR

1. To perform semi-nested PCR, two successive PCR reactions are needed.
2. Proceed with the initial reaction with the external primers of the particular region (in gene) of TiLV to yield primary amplicon.
3. Proceed with the successive reaction with either the initial forward or reverse primer used in the first reaction with an opposing internal primer within the same region.
4. Use this method for both fresh and preserved TiLV fish samples [10].
5. This method gives more specific results for the detection of positive TiLV samples [11].
6. The diagrammatic representation of nested and semi-nested PCR is shown in Fig. 3 [8].

3.4.3 Quantitative Real-Time PCR for TiLV Diagnosis

1. Real-time PCR is considered highly advantageous than other PCR methods due to its specificity and sensitivity [12].
2. Use this method for simultaneous detection and quantification of the DNA by measuring the fluorescent reporter.

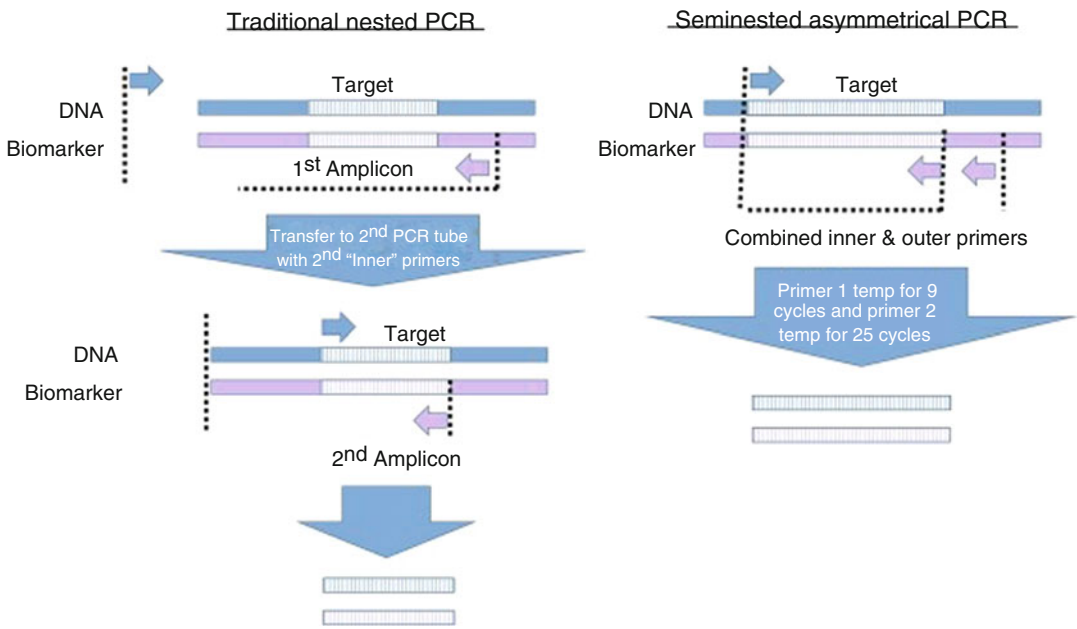

Fig. 3 Diagrammatic representation of nested PCR and semi-nested PCR assay for the specific diagnosis of TiLV

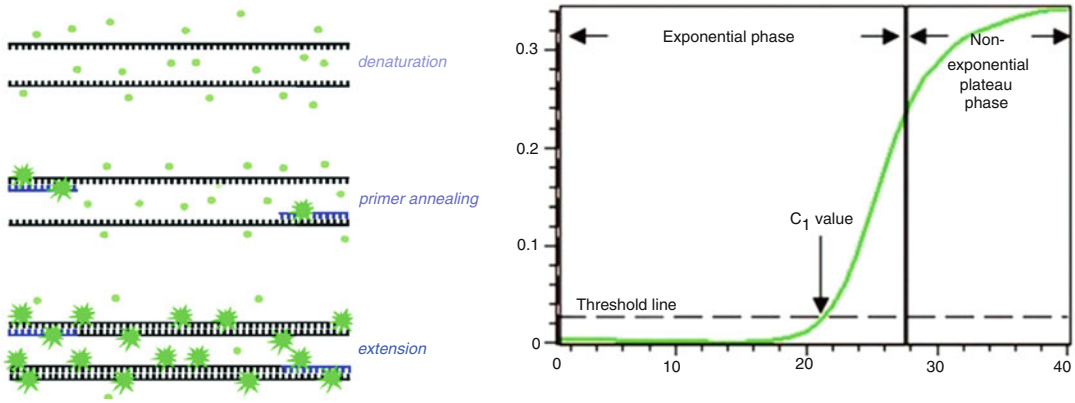

Fig. 4 Mechanism of real-time PCR with SYBR Green dye

3. When the DNA binds with the dye, it emits the light signal at excitation point as shown in Fig. 4.
4. As PCR product increases, the fluorescent signal increases which is recorded by the system for the result analysis.
5. Especially, this method is suitable for the detection of the viruses with its quantitative nature, its scalability, and its rapid time to result.
6. This method never need gel electrophoresis method of analysis [13, 14].

3.4.4 RT-LAMP

1. The loop-mediated isothermal amplification (LAMP) assay is used to detect viral RNA.
2. Two pairs of primers are used in this assay, one pair of inner primers and one pair of outside primers. These primers were designed specifically for the reaction.
3. To evaluate viral content, similar approaches rely on the turbidity of the sample, which increases as the amount of genetic material increases.
4. To amplify and detect the results, agarose gel electrophoresis method is used.
5. RT-LAMP is more sensitive than standard real-time PCR tests.
6. The results of this assay are more specific with nil false positivity [15].
7. For detecting more specific TiLV-positive samples, this assay is used with two different set of primers specific to the particular region in TiLV [16].

3.4.5 In Situ Hybridization

In situ hybridization technique is used for the accurate detection and recognition of nucleic acid sequence within a cell.

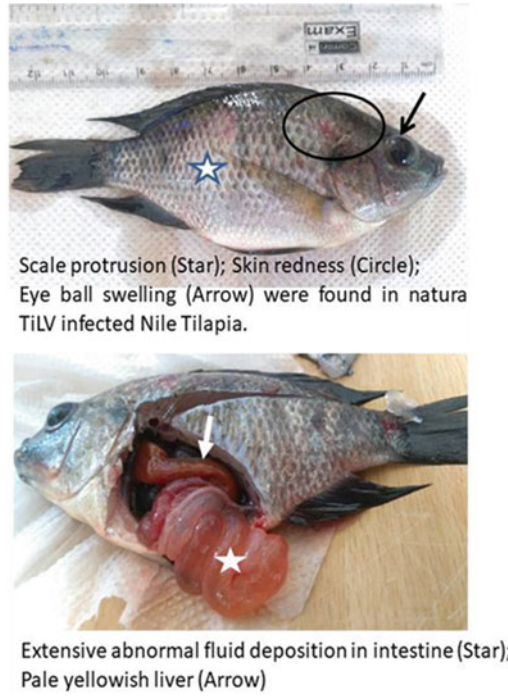

Fig. 5 Clinical signs of TiLV in tilapia fishes [6]

1. Complementary base pairing (hybridization) with a detectable nucleic acid segment (probe) binds with the nucleic acid sequence explicitly in a tissue section.
2. In situ hybridization (ISH) technique has three major benefits of high sensitivity and accurate anatomical localization and quantification [17].

3.5 Morphological Examination of TiLV

The preliminary examination of TiLV involves clinical signs by phenotypic observation. TiLV-infected fish tissue can be examined for histopathological changes and transmission electron microscope (TEM).

3.5.1 Clinical Observation

Diseased fish with TiLV has the following morphological changes which is shown in Fig. 5:

1. Ocular alterations
2. Skin erosions resulting in hemorrhagic dermal lesions (Eyngor et al., 2014)
3. Discoloration (darkening)
4. Abdominal distension (due to fluid or enlargement of spleen and other organs)
5. Exophthalmia [18]
6. Gill pallor [19]

7. Scale protrusion
8. Abnormal behavior [6]
9. Loss of appetite and lethargy [20].

3.5.2 Histology

1. Dissect the organs of the infected fish and preserve in 10% formalin for 24–36 h.
2. Briefly, dehydrate the specimen, and embed in paraffin to section at 5 μm.
3. Stain the thin section with hematoxylin and eosin (H&E).
4. Finally, examine the stained section under light microscope.
5. The pathological changes of TiLV in fish involve the following:
   - Necrosis of hepatocytes
   - Presence of brown lipoproteinaceous material (ceroid) within the hepatocytes
   - Stomach showing significant loss of gastric glands [19]

3.5.3 Transmission Electron Microscope

1. Dissect the tissue from the infected fish and preserve it in 2% glutaraldehyde in a cacodylate buffer.
2. Add 1% osmium tetroxide and resin embedding for ultrathin sectioning.
3. Stain this thin section with uranyl acetate and lead citrate and examine with transmission electron microscope (TEM).
4. The electron microscope can visualize multinucleated hepatocyte, appearance of viruslike particle within cytoplasm, and swelling of mitochondria and loss of cristae in the organs of fish infected with TiLV [19].

References

1. Elangovan P et al (2018) Cage culture of tilapia with respect to nutrition and feeding. J Aquac Trop 33:19. https://doi.org/10.32381/jat.2018.33.1-2.3
2. Jansen MD, Mohan CV (2017) Tilapia lake virus (TiLV): literature review. 2017
3. Bacharach E et al (2016) Characterization of a novel orthomyxo-like virus causing mass die-offs of Tilapia. MBio 7:e00431. https://doi.org/10.1128/mBio.00431-16
4. Eyngor M et al (2014) Identification of a novel RNA virus lethal to tilapia. J Clin Microbiol 52:4137. https://doi.org/10.1128/JCM.00827-14
5. Jaemwimol P, Rawiwan P, Tattiyapong P, Saengnual P, Kamlangdee A, Surachetpong W (2018) Susceptibility of important warm water fish species to tilapia lake virus (TiLV) infection. Aquaculture 497:462. https://doi.org/10.1016/j.aquaculture.2018.08.028
6. Saranya SR, Sudhakaran R (2020) Report on prevalence of tilapia lake virus infection in tilapia fishes (Oreochromis niloticus). Biocatal Agric Biotechnol 27:101665. https://doi.org/10.1016/j.bcab.2020.101665
7. Behera BK et al (2018) Emergence of Tilapia Lake Virus associated with mortalities of farmed Nile Tilapia Oreochromis niloticus (Linnaeus 1758) in India. Aquaculture 484:168. https://doi.org/10.1016/j.aquaculture.2017.11.025
8. Tsofack JEK et al (2017) Detection of tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. J Clin

Microbiol 55:759. https://doi.org/10.1128/JCM.01808-16

9. Carr J, Williams DG, Hayden RT (2010) Molecular detection of multiple respiratory viruses. In: Molecular diagnostics. Elsevier/Academic Press, Amsterdam
10. Mushtaq Z, Qayoom U, Mir IN, Mir S (2018) Tilapia lake virus: an emerging viral disease of tilapia industry. J Entomol Zool Stud 6(5): 141–144
11. Levin RE, Ekezie F-GC, Sun D-W (2018) DNA-based technique: polymerase chain reaction (PCR). In: Modern techniques for food authentication, pp 527–616. https://doi.org/10.1016/B978-0-12-814264-6.00014-1
12. Waiyamitra P, Tattiyapong P, Sirikanchana K, Mongkolsuk S, Nicholson P, Surachetpong W (2018) A TaqMan RT-qPCR assay for tilapia lake virus (TiLV) detection in tilapia. Aquaculture 497:184. https://doi.org/10.1016/j.aquaculture.2018.07.060
13. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol 34:597. https://doi.org/10.1677/jme.1.01755
14. Mackay IM (2004) Real-time PCR in the microbiology laboratory. Clin Microbiol Infect 10:190. https://doi.org/10.1111/j.1198-743X.2004.00722.x
15. Huang WE et al (2020) RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. Microb Biotechnol 13:950. https://doi.org/10.1111/1751-7915.13586
16. Yin J et al (2019) Development of a simple and rapid reverse transcription–loop mediated isothermal amplification (RT-LAMP) assay for sensitive detection of tilapia lake virus. J Fish Dis 42:817. https://doi.org/10.1111/jfd.12983
17. Laurent-Huck FM, Felix JM (1991) Measurement of oxytocin and vasopressin gene expression by in situ hybridization. Methods Neurosci 5, no. C:159–182. https://doi.org/10.1016/B978-0-12-185259-7.50015-6
18. Dong HT, Ataguba GA, Khunrae P, Rattanarojpong T, Senapin S (2017) Evidence of TiLV infection in tilapia hatcheries in Thailand from 2012 to 2017 reveals probable global spread of the disease. Aquaculture. https://doi.org/10.1016/j.aquaculture.2017.06.035
19. Ferguson HW, Kabuusu R, Beltran S, Reyes E, Lince JA, del Pozo J (2014) Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. J Fish Dis 37:583. https://doi.org/10.1111/jfd.12142
20. Surachetpong W, Roy SRK, Nicholson P (2020) Tilapia lake virus: the story so far. J Fish Dis 43:1115. https://doi.org/10.1111/jfd.13237

Chapter 9

Isolation and Identification of Pathogens from Shrimp: IHHNV

V. M. Amrutha, R. Bharath, K. Karthikeyan, R. Vidya, and R. Sudhakaran

Abstract

Aquaculture is the rapidly growing food sector around the globe; seafood is healthier and helps to treat more diseases. Unfortunately, the viral disease outbreak will result in economic losses and more harm to aquatic animals. Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is a non-enveloped single-stranded DNA virus. It belongs to the *Parvoviridae* family commonly detected in penaeid shrimp culture, infecting juvenile and post-larvae of giant aquatic shrimps. It causes runt-deformity syndrome characterized by stunted growth, physical abnormality, and cuticular deformities of the antenna, rostrum, and thoracic and abdominal area. In this study, we explained the existing procedures to isolate and identify the presence of IHHNV

Key words IHHNV, DNA extraction, Shrimp, Virus, PCR, Histopathology

1 Introduction

In recent years, the aquaculture industry is expanding considerably. Aquaculture, also known as aquafarming, is a complete artificial farming setup for the growth of fish, shrimp, medicinal plants, and other marine living organisms on land, mostly in tanks and ponds [1]. Onshore aquaculture is defined as culture that occurs near sea water handled by humans, inshore aquaculture is defined as culture that occurs in open water and growth occurs in a naturalistic environment [2], and offshore aquaculture is defined as culture that occurs in open water and growth occurs in a natural environment [3, 4]. Some types of aquaculture, such as shrimp and fish farming, do additional damage to ocean resources, such as waste disposal, exotic species, invasion of pathogens in species, and human demand for fish oil as medicine, which may deplete stocks [5]. Extensive aquaculture is characterized by the elimination of predators and the control of rivals; semi-intense aquaculture is characterized by the increased availability of food; and intensive

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_9,

aquaculture is characterized by the provision of all nutritional requirements [6].

Aquaculture is fast growing in food supply and medicinal sector; seafood are healthier and also can fight with diseases like cardiovascular diseases, cancer, and Alzheimer's disease and many other illnesses [7, 8]. In the coming decades, not only should fish output improve but also food safety and security, and aquatic organisms should be readily available, affordable, and accessible to people from all sectors [9, 10]. Flagella, yeasts, bacteria, and fungi can protect fish from disease while also providing nutrition similar to fish meal [11, 12]. Pathogenic microbes that will live feed the larvae stage of fishes will get more microbial infection than adults [13, 14].

IHHNV (infectious hypodermal and hematopoietic necrosis virus) is a viral pathogen non-enveloped single-stranded DNA virus; it belongs to the *Parvoviridae* family that affects aquatic creatures such as shrimp and crabs. Because these viruses constitute a constant threat to aquatic organisms, it is critical to understand the route of infection and the kind of transmission [15] of these viruses. In terms of host ranges and virulence, DNA virus has at least three strains; these strains have a greater host survival rate and a lower viral burden in the aquaculture setting. IHHNV will spread horizontally through water content and cannibalism, as well as vertically from the mother to the fetus [16]. The presence of IHHNV in shrimp farming was widely seen in post-larvae that is brought in from wild broodstock. Their clinical sign is a stunted rostrum that seems twisted to one side and a crooked body; these deformed shrimps account for 10–20% of the population [17]. This IHHNV has a moderate growth rate, has a poor survival rate, and is weak but not fatal. These viruses are found in countries such as India, Indonesia, Taiwan, etc. and infect species such as *P. japonicus* and *P. monodon*. This virus causes acute epizootics and enormous mortalities seen in juvenile and post-larvae of penaeid shrimp; however, *L. vannamei* growth is reduced and shrimp are small in size; therefore, there is no death [18]. Infected ectodermal and mesodermal tissues such as gills, cuticular epithelium, hematopoietic tissues, cord nerve, lymphoid organ, and antennal gland disseminate by crustaceans and vectors as shown in Fig. 1 [19, 20].

2 Materials

2.1 PCR Mixture

Tris–HCl, KCl, Triton X, each dNTPS, MgCl2, primers, extracted DNA, and Taq DNA polymerase.

2.2 Agarose Gel Preparation

0.8% of agarose is added to TBE buffer.

Fig. 1 IHHNV-infected shrimp's growth retardation [21]

2.3 Lamp PCR

Template, each primer, DNA polymerase, reaction buffer, dNTPs, distilled water to make up 50 μL, perform in one reaction tube.

2.4 G.HCl Preparation

10 mM Tris–HCl, 0.1 mol/L of sodium acetate, 6 mol/L G.HCl, 0.5 M of EDTA.

2.5 TBE Buffer

Trizma base, boric acid, EDTA.

Homogenizing sticks, ethanol, hematoxylin and eosin–phloxine, minimum essential medium (MEM), fetal bovine serum, glutaraldehyde, sodium chloride, sucrose, Eppendorf.

3 Extraction of IHHNV from Penaid Shrimp

3.1 DNA Extraction

- The extraction of DNA is done from extracting 100 mg of shrimp muscle using guanidine hydrochloride (G.HCl) method [22].
- 1000 μL of G.HCl in 100 mg of tissue homogenate. Incubate the sample for 30 min at room temperature. Centrifuge it for 5 min at 5000 rpm.
- Transfer the aqueous solution to fresh microcentrifuge tube and ethanol same amount. Incubate the sample at room temperature for 10 min and then centrifuge the sample for 15 min at 10,000 rpm.
- Discard the supernatant and add 95% ethanol 500 μL to the pellet, and centrifuge it for 10 min at 10,000 rpm.
- Discard the supernatant and add 500 μL of 75% ethanol to the pellet and then centrifuge it for 10 min at 10,000 rpm.
- Discard the supernatant and air-dry the pellet in room temperature and add 50 μL of sterile water [23].

Table 1
PCR primer

S. no	Type	Primer name	Primer sequence	Product length	Nucleotide position
1a	PCR	389F	5′-CGGAACACAACCCGACTTTA-3′	389 bp	1400–1788
		389R	5′-GGCCAAGACCAAAATACGAA-3′	389 bp	1400–1788
1b	Real-time PCR	IHHNV1608F	5′-TAC-TCC-GGA-CAC-CCA-ACC-A-3′	–	1632–1664
		IHHNV1688R	5′-GGC-TCT-GGC-AGC-AAA-GGT-AA-3′	–	1632–1664
		TaqMan probe	5′-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3′	–	1632–1664
1c	Nested PCR	IHHNV648F	5′-GAACGGCTTTCGTATTTTGG-3′	648 bp	–
		IHHNV648R	5′-AGCGTAGGACTTGCCGATTA-3′	648 bp	–

3.2 Polymerase Chain Reaction (PCR) Amplification

- The reaction mixture contains 10 mM of Tris–HCl, 50 mM KCl, 0.1% Triton X, 200 μM of each dNTPS (dATP, dGTP, dCTP, and dTTP), 2 mM MgCl2, 0.3 μM of primers (designed according to sequence which contains IHHNV infection as shown in Table 1a) [24], 1 μL of extracted DNA, and 0.625 U Taq DNA polymerase, and the final volume should be 50 μL.
- PCR amplification is performed: denaturation for 5 min at 94 °C for 35 cycles, annealing at 94 °C for 30 s, extension at 55 °C for 30 s, and final extension at 72 °C for 7 min held at 4 °C shown in Table 1a [25].

3.3 Post-PCR Analysis

- Agarose gel electrophoresis is done to visualize 1.6 kb amplifies product.
- 10 μL of PCR sample analyzed on 0.8% of agarose gel mixed with 0.5× Tris–Borate–EDTA (TBE) buffer and attain with ethidium bromide.
- Load a marker, positive control, negative control, and sample in agarose gel; run the gel electrophoresis.
- DNA is negative so it moves to the positive side when electrophoresis is done.
- Visualize under gel documentation system if the band is visible in specific base pair and then samples which are infected by IHHNV [26].

3.4 Sequence Comparison and Phylogenetic Analysis

- The nucleotide and deduced amino acid sequence of presumptive IHHNV DNA is compared to the existing IHHNV sequence in the National Center for Biotechnology Information (NCBI) using BLAST search, indicating that the sequence is located inside the nonstructural protein coding region of IHHNV [27].
- Clustal X to execute multiple alignment sequences and MEGA software can create a phylogenetic tree with the highest parsimony [28].

3.5 Real-Time PCR

- Real-time PCR is a technique used to quantify the nucleic acids like DNA and RNA presented in the sample during the PCR reaction, and it is also called quantitative PCR (qPCR).
- IHHNV is detected using PCR primers, and a TaqMan probe for a specific area of the IHHNV genomic sequence that codes for nonstructural protein is designed as shown in Table 1b [29].
- The upstream and downstream primer sequences are developed with IHHNV infection in sequence, and the TaqMan probe should contain nucleotides from 1632 to 1664 that generate and label with fluorescent dye.
- 10 ng of DNA template is added to a 25 μL PCR mixture with 0.3 M of upstream and downstream primers and 0.15 M of TaqMan probe [30].
- The ampliTaq is activated, and denaturation occurs for 10 min at 95 °C, followed by a 40-cycle response annealing for 15 s at 95 °C and extension for 1 min at 60 °C. The sample is tested in duplicate and results are considered positive [31].

3.6 Nested PCR

It is modification of polymerase chain reaction that reduces nonspecific binding in products due to the amplification of unexpected primer binding site.

- The primer is considering sequence related to nonstructural protein; DNA amplification is done by preheating for 3 min at 94 °C and 40-cycle denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s, and extension at 72 °C for 45 s after amplification with primer as shown in Table 1c; the amplified fragments are visualized in agarose gel electrophoresis stained with ethidium bromide [32].
- If IHHNV is transferred vertically through contaminated egg, we can perform nested PCR by extracting DNA from hemolymph in female shrimp; the IHHNV-positive samples are identified through nested PCR [33].

Table 2
LAMP reaction

Primer	Type	Sequence (5′–3′) a
IHHNV-F3	F3	CGACATCCGTGTACCAGA
IHHNV-B3	B3	AGAGCGTAGGACTTTCCG
IHHNV-FIP	F2-F1c	GTCCTTGGAGTACAAGAGTGTTTATGGATCCAATC TTAGCTTGGATAATCATCGT
IHHNV-BIP	B1c-B2	GAAAATCTCTTACCATCGGTGCAGGATCCGAAGGT GTTTGAGTCTCCT

3.7 LAMP Reaction

Loop-mediated isothermal amplification (LAMP) is a single-tube technique for the amplification of nucleic acids, and it is a low-cost alternative method to identify certain disease. It is also an isothermal amplification technique which can carry out alternative temperature.

- Forward inner primer (FIP), backward inner primer (BIP), and two outer primers are created specifically for monoplex LAMP techniques [34].
- Six different nucleotide sequences are amplified using four different primer target sequences at a constant temperature of 60–65 °C [35].
- To determine the temperature, the sample is incubated at various temperatures for 60 min and then terminated at 80 °C for 5 min with primer shown in Table 2, after which the amplified DNA was 2 μL template, 2 μL of each primers, 2 μL of DNA polymerase, 5 μL of reaction buffer, 2 μL of dNTPs, and 17 μL of distilled water to make up 50 μL. It is performed in one reaction tube exposed to electrophoresis [36].

3.8 Virus Isolation by Cell Culture

Cell culture is performed by removal of infected tissues from the infected animals and subsequently allowed to grow in artificial favorable conditions.

- In a tissue culture container, a single layer of epithelioma papulosum cyprinid cells is cultured in minimum essential medium (MEM) with 10% fetal bovine serum at 20 °C [37].
- An infected tissue is incubated for 1 h at room temperature in a 0.5 mL sample generated from infected shrimp tissues.
- MEMSFBS is added to the medium, and the container is incubated at 20 °C and inspected daily. The EPC cells which are not infected are retained as a control [38].

- The virus precipitates from supernates at 4 °C for 90 min with PEG to final concentration, centrifuge at 3000 *g* for 15 min at 5 °C, and resuspend in TNE buffer.
- The infected cell is freeze-thawed once and centrifuge at 3000 g for 15 min at 5 °C.
- This suspension is added to a 60% sucrose solution at 240,000 for 30 min at 5 °C. This tube's bluish viral band is collected and kept at −70 °C [39].

3.9 Histopathology

Histopathology is the study of tissue disease and it entails studying tissue under a microscope.

- Shrimp at moribund stage is collected from aquaria and transverse cut made in-between posterior end of cephalothorax and first abdominal segment the cephalothorax fixed, which is preserved in alcohol formalin acetic acid (AFA) [40].
- The specimen is transported to 70% ethanol and embedded in paraffin blocks after 24–48 h.
- For histological investigation, the sectioned shrimp tissue is stained with hematoxylin and eosin–phloxine [41].

3.10 In Situ Hybridization

In situ hybridization is a technique that allows for the detection of a specific nucleic acid segment from a histologic slice by using a complementary strand of nucleic acid to which molecules are bound [42].

- The reporter molecules allow nucleotide sequences to be localized in a cell population. A 4-m-thick paraffin piece is placed on a positively charged microscope slide and deparaffinized by heating it for 45 min at 65 °C [43].
- It is then rehydrated and begins to react with a DIG-labeled probe, yielding a dark-blue to purple precipitate, indicating the presence of IHHNV infection [31].

3.11 TEM Analysis

The transmission electron microscope (TEM) provides a highly enlarged image by using an electron particle beam that passes through the material [44].

- Individual organs, such as the heart, muscle, and hepatopancreatic tissues, are fixed using 6% glutaraldehyde, 1% sodium chloride, and 0.5% sucrose.
- The infected tissue is placed in 1 mL of fixative for 6 h at 4 °C, after which it is examined under a TEM [45].

References

1. Yeh SP, Chen YN, Hsieh SL, Cheng W, Liu CH (2009) Immune response of white shrimp, Litopenaeus vannamei, after a concurrent infection with white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus. Fish Shellfish Immunol 26(4): 582–588. https://doi.org/10.1016/j.fsi.2008.09.010
2. Hoang MN, Nguyen PN, Bossier AMVEM, Bossier P (2021) Effects of shrimp-fish polyculture on immune parameters, disease resistance of white shrimp and the prevalence of Vibrio spp. Aquac Res. https://doi.org/10.1111/ARE.15666
3. Rico A et al (2013) Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquaculture 412–413: 231–243. https://doi.org/10.1016/J.AQUACULTURE.2013.07.028
4. Naylor RL et al (2000) Effect of aquaculture on world fish supplies. Nature 405(6790): 1017–1024. https://doi.org/10.1038/35016500
5. Loh PC, Lu Y, Brock JA (1990) Growth of the penaeid shrimp virus infectious hypodermal and hematopoietic necrosis virus in a fish cell line. J Virol Methods 28(3):273–280. https://doi.org/10.1016/0166-0934(90)90120-5
6. Bostock J et al (2010) Aquaculture: global status and trends. Philos Trans R Soc B Biol Sci 365(1554):2897–2912. https://doi.org/10.1098/RSTB.2010.0170
7. Owens L et al (1992) Infectious hypodermal and haematopoietic necrosis virus (IHHNV) in a hybrid penaeid prawn from tropical Australia. Dis Aquat Org 14:219–228
8. Finegold C (2009) The importance of fisheries and aquaculture to development. Ksla 484 [Online]. Available https://digitalarchive.worldfishcenter.org/handle/20.500.12348/1445. Accessed 25 Nov 2021
9. Subasinghe RP. An outlook for aquaculture development: major issues, opportunities and challenges 1
10. Aquaculture: challenges and promise | learn science at scitable. https://www.nature.com/scitable/knowledge/library/aquaculture-challenges-and-promise-23690921/. Accessed 26 Nov 2021
11. Marchini, A, Ferrario J, Occhipinti A (2016) The relative importance of aquaculture AND shipping as vectors of introduction of marine alien species: the case of olbia (sardinia) [Online]. Available https://www.researchgate.net/publication/311600252. Accessed 25 Nov 2021
12. Bentzon-Tilia M, Sonnenschein EC, Gram L (2016) Monitoring and managing microbes in aquaculture – towards a sustainable industry. Microb Biotechnol 9(5):576–584. https://doi.org/10.1111/1751-7915.12392
13. Caruso G (2014) Effects of aquaculture activities on microbial assemblages. https://doi.org/10.4172/2332-2632.1000e107
14. Seibert CH, Pinto AR (2012) Challenges in shrimp aquaculture due to viral diseases: distribution and biology of the five major penaeid viruses and interventions to avoid viral incidence and dispersion. *Braz J Microbiol* 43(3): 857–864
15. Macías-Rodríguez NA et al (2014) Prevalence of viral pathogens WSSV and IHHNV in wild organisms at the Pacific Coast of Mexico. J Invertebr Pathol 116(1):8–12. https://doi.org/10.1016/J.JIP.2013.11.002
16. Doyle RW (2005) Breeding shrimp for disease resistance: challenges and opportunities for improvement [Online]. Available https://www.researchgate.net/publication/265990674. Accessed 28 Nov 2021
17. IHHNV infection from the wild shrimps of Andaman and Nicobar Islands, India on JSTOR. https://www.jstor.org/stable/26494852. Accessed 30 Nov 2021
18. Gunalan B, Soundarapandian P, Anand T, Kotiya AS, Simon NT (2014) Disease occurrence in Litopenaeus vannamei shrimp culture systems in different geographical regions of India. Int J Aquac 4(0):24–28. https://doi.org/10.5376/ija.2014.04.0004
19. Dewangan NK et al (2017) Incidence of simultaneous infection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) and white spot syndrome virus (WSSV) in Litopenaeus vannamei. Aquaculture 471:1–7. https://doi.org/10.1016/J.AQUACULTURE.2017.01.002
20. Cavalli LS et al (2013) Natural occurrence of White spot syndrome virus and Infectious hypodermal and hematopoietic necrosis virus in Neohelice granulata crab. J Invertebr Pathol 114(1):86–88. https://doi.org/10.1016/J.JIP.2013.06.002
21. Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) education series [Online]. Available www.genics.com. Accessed 14 Nov 2022
22. Escobedo-Bonilla CM, Rangel JLI (2014) Susceptibility to an inoculum of infectious

hypodermal and haematopoietic necrosis virus (IHHNV) in three batches of whiteleg shrimp Litopenaeus vannamei (Boone, 1931). *Zookeys* 457(457):355. https://doi.org/10.3897/ZOOKEYS.457.6715

23. Pramanick D, Forstová J, Pivec L (1976) 4 M guanidine hydrochloride applied to the isolation of DNA from different sources. FEBS Lett 62(1):81–84. https://doi.org/10.1016/0014-5793(76)80021-X
24. Yang B, Song XL, Huang J, Shi CY, Liu L (2007) Evidence of existence of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp cultured in China. Vet Microbiol 120(1–2):63–70. https://doi.org/10.1016/J.VETMIC.2006.10.011
25. Tang KFJ, Poulos BT, Wang J, Redman RM, Shih HH, Lightner DV (2003) Geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. Dis Aquat Org 53(2):91–99. https://doi.org/10.3354/DAO053091
26. Nunan LM, Poulos BT, Lightner DV (2000) Use of polymerase chain reaction for the detection of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp. Mar Biotechnol 2(4):319–328. https://doi.org/10.1007/S101260000003
27. Lee C et al (2021) Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV, Decapod penstylhamaparvovirus 1) in commodity red claw crayfish (Cherax quadricarinatus) imported into South Korea. *J Mar Sci Eng* 9(8):856. https://doi.org/10.3390/JMSE9080856
28. Setälä O, Lehtiniemi M, Coppock R, Cole M (2018) Microplastics in marine food webs. In: *Microplastic contamination in aquatic environments: an emerging matter of environmental urgency*. Elsevier, pp 339–363
29. Detection of IHHNV in shrimp by dot blot hybridization with digoxigenin labeled DNA probe by PCR. J Fish Sci China 2004年02期. https://en.cnki.com.cn/Article_en/CJFDTotal-ZSCK200402002.htm. Accessed 29 Nov 29 2021
30. Dhar AK, Roux MM, Klimpel KR (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus and white spot virus in shrimp using real-time quantitative PCR and SYBR green chemistry. *J Clin Microbiol* 39(8):2835. https://doi.org/10.1128/JCM.39.8.2835-2845.2001
31. Tang KFJ, Lightner DV (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. Dis Aquat Org 44(2): 79–85. https://doi.org/10.3354/DAO044079
32. Non-structural Protein 1 (NS1) Gene
33. Motte E et al (2003) Prevention of IHHNV vertical transmission in the white shrimp Litopenaeus vannamei. Aquaculture 219(1–4): 57–70. https://doi.org/10.1016/S0044-8486(02)00631-2
34. He L, Xu HS (2011) Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp. Aquaculture 311(1–4):94–99. https://doi.org/10.1016/J.AQUACULTURE.2010.11.051
35. Kiatpathomchai W, Jaroenram W, Arunrut N, Jitrapakdee S, Flegel TW (2008) Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J Virol Methods 153(2):214–217. https://doi.org/10.1016/J.JVIROMET.2008.06.025
36. Sun ZF, Hu CQ, Ren CH, Shen Q (2006) Sensitive and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimps by loop-mediated isothermal amplification. J Virol Methods 131(1):41–46. https://doi.org/10.1016/J.JVIROMET.2005.07.011
37. Preliminary screening for infectious hypodermal and hematopoietic necrosis viruscapsid protein receptors from Litopenaeus vannamei gill membrane proteins. J Fish Sci China 2015年06期. https://en.cnki.com.cn/Article_en/CJFDTotal-ZSCK201506009.htm. Accessed 30 Nov 2021
38. Hou L, Wu H, Xu L, Yang F (2009) Expression and self-assembly of virus-like particles of infectious hypodermal and hematopoietic necrosis virus in Escherichia coli. Arch Virol 154(4): 547–553. https://doi.org/10.1007/S00705-009-0336-6
39. Lu Y, Cesar E, Nadala B, Brock JA, Loh PC (1991) A new virus isolate from infectious hypodermal and hematopoietic necrosis virus (IHHNV)-infected penaeid shrimps. J Virol Methods 31(2–3):189–195. https://doi.org/10.1016/0166-0934(91)90157-U
40. Mari J, Bonami JR, Lightner D (1993) Partial cloning of the genome of infectious hypodermal and haematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. J Gen Virol 74(12):2637–2643. https://doi.org/10.1099/0022-1317-74-12-2637/CITE/REFWORKS

41. Vanpatten KA, Nunan LM, Lightner DV (2004) Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. Aquaculture 241(1–4):31–46. https://doi.org/10.1016/J.AQUACULTURE.2004.08.012
42. Hsieh CY et al (2006) Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, Macrobrachium rosenbergii. Aquaculture 258(1–4): 73–79. https://doi.org/10.1016/J.AQUACULTURE.2006.04.007
43. Teng PH et al (2006) Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in Litopenaeus vannamei by ramification amplification assay. Dis Aquat Org 73(2):103–111. https://doi.org/10.3354/DAO073103
44. Zhu YP, Li C, Wan XY, Yang Q, Xie GS, Huang J (2019) Delivery of plasmid DNA to shrimp hemocytes by infectious hypodermal and hematopoietic necrosis virus (IHHNV) nanoparticles expressed from a baculovirus insect cell system. J Invertebr Pathol 166:107231. https://doi.org/10.1016/J.JIP.2019.107231
45. Chayaburakul K, Lightner DV, Sriurairattana S, Nelson KT, Withyachumnarnkul B (2005) Different responses to infectious hypodermal and hematopoietic necrosis virus (IHHNV) in Penaeus monodon and P. vannamei. Dis Aquat Org 67(3):191–200. https://doi.org/10.3354/DAO067191

Chapter 10

Isolation and Identification of *Ichthyophonus hoferi* from Fishes

Haimanti Mondal, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

Ichthyophonus hoferi is a fish pathogen found commonly in both freshwater and marine fishes. *I. hoferi* causes a granulomatous systemic infection, called ichthyophoniasis. There has been a lot of recorded evidence of this infection resulting into heavy mass mortalities of more than 80 freshwater and marine species, leading to huge economic losses. This parasite has been reported to produce infections in different species of marine, freshwater, and estuarine teleosts, adapting a wide range of environmental factors.

Key words *Ichthyophonus hoferi*, Systemic, Ichthyophoniasis, Granulomatous, Parasite

1 Introduction

Ichthyophonus hoferi is a single-celled protist having an amoeboid stage and hyphae. The cell wall is made up of chitin. It belongs to the class Ichthyosporea, order Ichthyophonida, and genus *Ichthyophonus* [1, 2]. In 1893, von Hofer first identified this parasite from cultured brook and brown trout in Germany. Later, in 1911, based on the nature of *I. hoferi*, it was named and described as a fungus by Plehn and Mulsow. Ultimately, it was classified under Mesomycetozoea [3, 4].

Zadeh et al. [4] conducted a study on the naturally infected freshwater. They detected *Ichthyophonus hoferi* from two ornamental fishes: black tetra, *Gymnocorymbus ternetzi*, and tiger barb, *Puntigrus tetrazona*. Kocan [5] reported a study on the transmission life cycle of *I. hoferi*. They revealed that it is still an important fish pathogen in both cultured and wild fishes.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_10,

2 Materials

- Tissue, kidney, liver, intestine, and spleen samples.
- Petri plates.
- Autoclave bags.
- Eagle's minimum essential medium (MEM).
- Sabouraud's dextrose agar (SDA).
- Giemsa stain.
- Trypticase soy agar (TSA) medium.
- Dorset egg medium.
- 10% phosphate-buffered natural formalin.
- Hematoxylin–eosin or periodic acid–Schiff stain.
- DNeasy tissue kit (Qiagen).
- Ethylenediaminetetraacetic acid (EDTA).
- NaCl–Tris–EDTA (NTE) buffer.
- DNA extraction buffer.
- Tris–HCl.
- Absolute ethanol.
- 70% ethanol.
- PCR primers.
- Dissecting needle.
- Sa-Iran camera microscope.
- Centrifuge.
- Speed Vac concentrator.
- Electrophoresis.
- UV transilluminator.

3 Methods

3.1 Isolation of Ichthyophonus hoferi *[4]*

3.1.1 Tissue Culture

- Take the tissue sample.
- Homogenize the samples in NTE buffer.
- Culture in MEM supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 IU/mL).
- Incubate the cultures at 25 °C and examine it daily.
- Prepare the dry smear of tissue after 2 weeks and stain with Giemsa stain.

3.1.2 Mycological and Bacteriological Examination

Bacteriological Examination

- Collect the samples from different organs—the spleen, kidneys, and liver.
- Cultivate on trypticase soy agar (TSA) medium at 32 °C for 48 h.
- Transport the colonies to Dorset egg medium.
- Incubate at 25 °C for 14 days.

Mycological Examination

- Take the samples from several internal organs like the liver, intestine, spleen, and kidney using a sterile dissecting needle.
- Inoculate onto SDA and MEM-10 medium with 1% fetal bovine serum.
- Incubate the plates and tubes at room temperature for 15 days.

3.2 Identification of Ichthyophonus hoferi

3.2.1 Microscopic Examination

- Identify the isolates by observing the morphological characteristics including the multinucleated spores, hyphal growth using stained preparation, and wet mount microscopical examination.

3.3 Polymerase Chain Reaction (PCR) Detection

3.3.1 Extraction of DNA

- Homogenize 50 mg of tissue sample in 100 µL of DNA extraction buffer.
- Dissolve the homogenized tissue or pellet in extraction buffer for 10 min at room temperature.
- Centrifuge at 5000 rpm for 5 min at 4 °C.
- Transfer 600 µL of supernatant in another tube.
- Add two volumes of 100% ethanol to precipitate the DNA.
- Mix the sample properly.
- Centrifuge at 12,000 rpm for 20 min at 4 °C to get the DNA pellet.
- Pour off the supernatant and wash the pellet with 70% ethanol.
- Dry the ethanol using Speed Vac concentrator.
- Dissolve the DNA in appropriate amount of distilled water or Tris–EDTA (TE) buffer.
- Store at 4 °C or −20 °C.

3.3.2 PCR Assay [6]

- Design the PCR primers—forward primer, Ich7f (5′-GCT -CTT-AAT-TGA-GTG-TCT-AC-3′), and reverse primer, Ich6r (5′-CAT-AAG-GTG-CTA-ATG-GTG-TC-3′).
- Prepare the reaction in 25 µL volume containing 1X PCR buffer, 0.2 mM dNTP, 2.5 Mm $MgCl_2$, 25 pmol of each primer, 2 µL template DNA, and 0.025 U/µL DNA *Taq* polymerase.

- Carry out the reaction for 35 cycles—initial denaturation at 95 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s, followed by final extension at 72 °C for 7 min.
- Run the electrophoresis with PCR products using agarose.
- Visualize under UV transilluminator.

References

1. Torruella G, Mendoza A, Grau-Bové X, Antó M, Chaplin MA, Campo J, Eme L, Pérez-Cordón G, Whipps CM (2015) phylogenomics reveals convergent evolution of lifestyles in close relatives of animals and fungi. Curr Biol 25(18): 2404–2410
2. Grau-Bové X, Torruella G, Donachie S, Suga H, Leonard G, Richards TA, Ruiz-Trillo I (2017) Dynamics of genomic innovation in the unicellular ancestry of animals. eLife 6:e26036
3. Kocan RM (2013) Proposed changes to the nomenclature of Ichthyophonus sp. life-stages and structures. J Parasitol 99:906–909
4. Zadeh MJ, Peyghan R, Manavi SE (2014) The detection of *Ichthyophonus hoferi* in naturally infected fresh water ornamental fishes. J Aquac Res Dev 5:289
5. Kocan RM (2018) Transmission models for the fish pathogen *Ichthyophonus*: synthesis of field observations and empirical studies. Can J Fish Aquat Sci 76:636–642
6. Whipps CM, Burton T, Watral VG, St-Hilaire S, Kent ML (2006) Assessing the accuracy of a polymerase chain reaction test for *ichthyophonus hoferi* in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Dis Auat Org 68:141–147

Chapter 11

Isolation and Identification of *Branchiomyces demigrans* from Fishes

Haimanti Mondal, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

For more than past two decades, aquaculture has been one of the most growing activities around the globe. *Branchiomyces demigrans* is one of the common devastating fungal pathogens, threatening several freshwater fishes and causing mortalities, leading to huge economic losses. The diameter of hyphae of *B. demigrans* usually ranges from 13–14 μm to 22–28 μm at the hyphal end. The wall thickness is 0.5–0.7 μm and the diameter of spore is approximately 12–17 μm. They are found in fishes with low pH, 5.8–6.5, low dissolved oxygen, and less stress.

Key words *Branchiomyces demigrans*, Hyphae, Dissolved oxygen, Stress

1 Introduction

B. demigrans is one of the fungal species responsible for causing *branchiomycosis*, infecting northern pike, large-mouth bass, striped bass, and tench. *Branchiomycosis* is a fungal disease common in fishes, especially in carp occurring almost all over the world. Sometimes, this pathogenic disease causes gill rot, leading to high mortality in huge populations of freshwater fishes. *B. demigrans* affects the entire gill region. The hyphae penetrate the wall of the blood vessels into the lumen and gill filaments, spreading on the surface [1].

Khalil et al. [2] reported a study on the *Branchiomyces demigrans* infection in common carp, *Cyprinus carpio* L., and Nile tilapia, *Oreochromis niloticus*, at different areas in Egypt, emphasizing on the environmental stress factor. They revealed the diameter of non-septated hyphae and spores. They described other fungal characteristics and the infection branchiomycosis caused by *B. demigrans*.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_11,

2 Materials

- Cotton swab.
- 70% ethyl alcohol.
- Petri plates.
- Autoclave bags.
- Lactophenol blue stain.
- Sabouraud's dextrose agar (SDA) medium (yeast extract, 5 g; distilled water, 1 L).
- Antibiotics—cycloheximide (0.5 g), gentamycin (0.65 mL from 40 mg/mL), chloramphenicol capsule (1 × 250 mg).
- 10% phosphate-buffered natural formalin.
- Eosin and hematoxylin stains.
- Ethylenediaminetetraacetic acid (EDTA).
- NaCl–Tris–EDTA (NTE) buffer.
- DNA extraction buffer.
- Tris–HCl.
- Absolute ethanol.
- PCR primers.
- Chloroform.
- Paraffin wax.
- Light microscope.
- Electrophoresis.
- UV transilluminator.

3 Methods

3.1 Isolation of Branchiomyces demigrans *[3–5]*

- Disinfect the fish tissue samples with a cotton swab and moisten with 70% ethanol.
- Homogenize the samples with NTE buffer.
- Culture the samples on SDA medium supplemented with cycloheximide, gentamycin, and chloramphenicol.
- Incubate the plates at 28 °C for 7 days.
- Subculture the colonies at 38 °C for 5 days.
- Stain with lactophenol cotton blue to examine the fungal growth on a clean slide.
- Observe the shape, color, texture, fungal growth of the colony, conidium, and spores, and measure the diameter of hyphae.

3.2 Identification of Branchiomyces demigrans *[6]*

3.2.1 Mycological Examination

- Mount the slides under light microscope.
- Examine the gills directly.
- Observe the spore characteristics of the *Branchiomyces demigrans*.

3.2.2 Histopathological Examination

- Fix the specimen in 10% phosphate-buffered natural formalin for 24 h.
- Wash it under running tap water.
- Dehydrate through ethanol.
- Clean it with chloroform and embed in paraffin wax at 60 °C.
- Take 5μm sections of the tissue. Stain with eosin and hematoxylin stains.

3.2.3 Morphometry of Gills

- Measure the length and breadth of lamella using a microscale meter.
- Estimate the number of colony-forming units/gram of weight (CFU/g) of fungal species.

3.2.4 Molecular Detection Using Polymerase Chain Reaction (PCR)

Extraction of DNA

- Homogenize 50 mg of tissue sample in 100 μL of DNA extraction buffer.
- Dissolve the homogenized tissue or pellet in extraction buffer for 10 min at room temperature.
- Centrifuge at 5000 rpm for 5 min at 4 °C.
- Transfer 600 μL of supernatant to another tube.
- Add two volumes of 100% ethanol to precipitate the DNA.
- Mix the sample properly.
- Centrifuge at 12,000 rpm for 20 min at 4 °C to get the DNA pellet.
- Pour off the supernatant and wash the pellet with 70% ethanol.
- Dry the ethanol using Speed Vac concentrator.
- Dissolve the DNA in appropriate amount of distilled water or Tris–EDTA (TE) buffer.
- Store at 4 °C or −20 °C.

PCR Assay

- Perform the PCR tests to detect the DNA in the samples.
- Design two universal primers, IST1 (5′-TCC-GTA-GGT-GAA-CCT-GCG-G-3′) and ISP4 (5′-TCC-TCC-GCT-TAT-TGA-TAT-GC-3′), for the fungal amplification.

- Carry out the reaction for 35 cycles—initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, and elongation at 72 °C for 30 s, followed by final elongation at 72 °C for 5 min.
- Run the electrophoresis with PCR products using 2% agarose.
- Visualize under UV transilluminator.

References

1. Mahboub HH, Shaheen AA (2019) Mycological and histopathological identification of potential fish pathogens in Nile tilapia. Aquaculture 530: 735849. https://doi.org/10.1016/j.aquaculture.2020.735849
2. Khalil RH, Saad TT, Selema TAM, Latif HMR (2015) *Branchiomyces demigrans* infection in farm-reared common carp (*Cyprinus carpio L.*) and nile tilapia, (*Oreochromis niloticus*) at different localities in Egypt, with special emphasis to the role of environmental stress factors. Int J Innov Stud Aquat Biol Fish 1(1):15–23
3. Adeshina I, Amoka PP, Tiamiyu LO, Abubakar M, Adesanmi O (2019) Natural formation and pathological effects of *Branchiomyces* Species in farm-grown African catfish *(Clarias gariepinus)* in Ilorin metropolis. Harran Üniv Vet Fak Derg 8(2):186–190
4. Ibrahim KS (2011) Isolation and pathological study of Branchiomycosis from the commercial pond of common carp *(Cyprinus carpio)* fish, in Governorate of Duhok/Iraq. Iraqi J Vet Med 35(1):1–9
5. Alfisyahrin F, Prayitno SB, Sarjito (2019) Prevalence and identification of fungal disease on Catfish *(Clarias gariepinus)* juvenile at Kendal Coastal region, Central Java. IOP Conf Ser Earth Environ Sci 246:012048
6. Bouhy ZM, Shaheen AA, Hassanin ME, Mahboub HH (2014) Branchiomycosis in nile tilapia *(Oreochromis niloticus)* in Behiera governorate with trials for treatment. Zag Vet J 42(3):29–42

Chapter 12

Isolation and Identification of Microsporidian Parasites, *Enterocytozoon hepatopenaei* Infection of Penaeid Shrimp

K. Karthikeyan, V. M. Amrutha, S. R. Saranya, and R. Sudhakaran

Abstract

In shrimp cultures, the microsporidian parasite *Enterocytozoon hepatopenaei* infection has become a well-known disease with a critical need to appeal to the sector. Recently, there have been considerable economic difficulties in the shrimp farming industry. Early diagnosis and quarantine is the most desirable and current practice because it does not have an adequate treatment or prophylactic protocol for infection. In recent days, various molecular-based diagnosis approaches have been reported. In this study, we describe a molecular-based technique to diagnosing EHP infection in shrimp culture.

Key words EHP, Shrimp, Real-time PCR, Staining, Microscope, Diagnosis

1 Introduction

Aquaculture is the fastest expanding food sector in the world, with countries all over the world cultivating shrimp and prawns. In Asia, the genus *Penaeus* is mostly employed for agriculture. *Penaeus monodon* is easy to domesticate in cultivation. Later, *P. vannamei* became close to shrimp producers worldwide because it can withstand pathogenic circumstances and survive, unusually. White spot disease (WSD) is a serious pathogen in shrimp farming history, yet this species has avoided epidemics [1].

A statistical report from the Marine Products Export Development Authority (MPEDA) observed remarkable growth in global shrimp exports after the introduction of this non-native species [2]. Shrimp cultivation and export have rapidly increased in India. These activities are often negatively influenced by factors such as improper management, environmental stress, and different diseases. Various pathogens, such as protozoa, fungi, bacteria, and viruses, from the natural environment cause significant disease outbreaks.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_12,

In pond-reared *L. vannamei* in various parts of India, a disease caused by a novel microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP), has been observed, according to [3]. This parasite was discovered to be responsible for significant economic loss in the shrimp culture business by slowing shrimp growth in culture ponds, and the loss persists to this day [4]. This parasite was discovered and characterized in 2009 [3], followed by an increase in the occurrence of microsporidian illness in a number of nations [5] [6].

2 Materials and Methods

2.1 Isolation of Microsporidian Spores from Shrimp Hepatopancreas and Fecal Matters [7]

- Carefully dissect the EHP-infected shrimp and retrieve the hepatopancreas without disturbing the stomach region to avoid microbial contaminations. For the extraction of spores from fecal matter: Collect the feces of EHP-infected shrimp.
- Using 500 µL of dimethyl ether, homogenize the shrimp hepatopancreas and fecal matter and continuously vortex the mixture for 1 min.
- Centrifuged for 5 min at 5000 rpm, then decant the supernatant, and save the pellet.
- To wash the pellet, add 500 µL of sterile distilled water and centrifuge it as described above. Repeat the washing process twice more.
- Collect the pellet containing partly purified EHP spores and store it at −80 °C.

2.2 Identification: Molecular Methods

Total DNA extraction from EHP shrimp tissue using standard phenol chloroform method:

2.2.1 DNA Extraction

- Weigh 100 mg EHP suspect shrimp tissue and add 500 µL of lysis buffer (50 mM Tris–HCl pH 9, 0.1 M EDTA pH 8, 50 mM NaCl, 2% SDS).
- Homogenize it completely and add 5 ug of proteinase K enzyme and incubate at 55°C for 30 min.
- Subsequently, add equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuge at 16,000 × *g* for 5 min.
- Collect the aqueous layer and transfer it into fresh 1.5 mL micro centrifuge tube.
- Add equal volume of ice-cold 100% ethanol and incubate it at −80°C/−20°C for 1 h and centrifuge at 16,000 × *g* for 15 min at 4 °C.
- Add 500 µL of 70% ethanol and centrifuge at 16,000 × *g* for 5 min and decant the supernatant and dry the pellet.

- Resuspend the DNA pellet in sterile water and store at −20 °C for further analysis.

Conventional Polymerase Chain Reaction: [6, 8].

2.3 Amplification of EHP Targeted Genome Will Perform in Thermal Cycler. The Standard Conventional PCR 20 μL Reaction (Karthikeyan et al. [10], FTA)

- Add 2 μL of 10× 15 mM $MgCl_2$ buffer into 0.2 mL PCR tubes.
- Add 0.5 to 2.5 U of *Taq* DNA polymerase (as per manufacture recommendation).
- 200 μM of each of the four nucleotides (200 μM dNTPs).
- 50–200 μM of each forward and reverse primers.
- Add 10–100 ng of total genomic DNA.
- Make up the volume up to 20 μL by addition of sterile PCR water.
- Thermal cycler conditions are as follows: initial denaturation at 95 °C for 3–5 min, subsequent 35 cycles of denaturation at 95 °C for 10–60 s, annealing at 50–65 °C for 5–60 s, extension at 68–72 °C for 20–120 s, and single cycle of final extension at 68–72 °C for 3–10 min.
- Visualize the amplified PCR product by running in agarose gel electrophoresis and examine it under UV transillumination.

2.4 Quantitative Polymerase Chain Reaction [9, 10]

Absolute quantification target EHP genomic DNA estimation upon the known concentration of EHP DNA:

- Add 12.5 μL of 2× SYBR Green I (user selection preference) master mix (Taq HS DNA polymerase, dNTP mixture, Mg2+, RNaseH, and SYBR Green I), 50–200 pmol each forward and revere primer.
- Add genomic DNA from 10 to 100 ng and 1 μL of serially dilute EHP plasmid.
- Thermal conditions are initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s (follow manufacture instruction).
- For quantitative analysis, interpret the results according to the standard curve (known plasmid copies) with unknown samples; for detection assay, interpret with the cycle threshold values.

2.5 Identification: Microscopy Examination

2.5.1 Hematoxylin and Eosin Stain [8]

- Fix the sample EHP-infected shrimp hepatopancreas in 10% formalin.
- Prepare a 3–5 um size section using microtome and clean the section with distilled water.
- Immerse section into hematoxylin containing coupling jar for 10 s.
- Remove the excess stain by allowing the slide in running tap water.

- Rinse the section with 0.3% acid alcohol until the background becomes colorless.
- Rinse the slide with running tap water.
- Immerse the section into eosin containing coupling jar for 30 s.
- Rinse the slide with running tap water.
- Dehydrate the section by applying ascending alcohol wash (50, 70, 80, 95, and 100%).
- Rinse with xylene for three to five times.
- Cover the section using coverslip with suitable mounting medium.

2.5.2 Modified Trichrome Stain [11]

- Prepare a smear of purified EHP spores on glass slide and allow to dry.
- Add drop of absolute methanol on the smear and allow for 5 min.
- Place the glass slide in filtered trichrome stain for 90 min.
- Rinse the excess stain using acid alcohol for not more than 10 s.
- Place the glass slide on 95% and 100% ethanol for 10 min each.
- Place the glass slide on xylene for 10 min.
- Mount with coverslip with suitable mounting medium.
- Examine the EHP spores under oil immersion.

2.5.3 Fluorescent Staining: Chitin Staining [12]

- Place a small piece of hepatopancreas on a 0.01% poly-lysine-coated slide.
- Add 50 µL of 4% paraformaldehyde and 50% Triton (49:1; *v/v*) and incubate it for 25 min at room temperature.
- Wash the slide with phosphate-buffered saline (pH 7.0) for three times.
- Add 50 µL of Fluorescent Brightener 28 (1:1000 dilution; Sigma, USA) and incubate it for 5 min at room temperature. Repeat wash step.
- Observe the EHP spores under fluorescent microscope.

2.5.4 Calcofluor White Stain [13]

- Collect EHP-infected shrimp fecal sample/hepatopancreas and homogenate with phosphate-buffered saline using plastic homogenized sticks.
- Prepare a smear of small portion of homogenates on glass slide.
- Add drop of mixture of Calcofluor white stain and Evans blue stain and incubate it for 5 min.
- Observe the EHP spores under the fluorescent microscope.

2.5.5 Transmission Electron Microscopy (TEM) [11]

- Place a drop of purified spores on carbon-coated 200 mm grid.
- Add a drop of 2% freshly prepared phosphotungstic acid and let it dry under hot plate (45 °C).
- Observe the EHP spore ultrastructural details under transmission electron microscope.

References

1. Thitamadee S et al (2016) Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture* 452:69–87. https://doi.org/10.1016/j.aquaculture.2015.10.028
2. Karthikeyan K, Sharma A, Mekata T, Itami T, Sudhakaran R (2017) Rapid and sensitive real-time loop meditated isothermal amplification for the detection of Enterocytozoon hepatopenaei of shrimp. *Aquaculture* 481 (September):119–123. https://doi.org/10.1016/j.aquaculture.2017.08.036
3. Tourtip S et al (2009) Enterocytozoon hepatopenaei sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp Penaeus monodon (Decapoda: Penaeidae): fine structure and phylogenetic relationships. J Invertebr Pathol 102(1):21–29. https://doi.org/10.1016/j.jip.2009.06.004
4. Newman SG (2015) Microsporidian impacts shrimp production. Glob Aquac Advocate March/April(September):33–35
5. Tang KFJ, Pantoja CR, Redman RM, Han JE, Tran LH, Lightner DV (2015) Development of in situ hybridization and PCR assays for the detection of Enterocytozoon hepatopenaei (EHP), a microsporidian parasite infecting penaeid shrimp. J Invertebr Pathol 130:37–41. https://doi.org/10.1016/j.jip.2015.06.009
6. Kesavan K, Mani R, Toshiaki I, Sudhakaran R (2017) Quick report on prevalence of shrimp microsporidian parasite Enterocytozoon hepatopenaei in India. Aquac Res 48(7): 3980–3984. https://doi.org/10.1111/are.13078
7. Bukhari Z, Smith HV (1995) Effect of three concentration techniques on viability of Cryptosporidium parvum oocysts recovered from bovine feces. J Clin Microbiol 33(10): 2592–2595. https://doi.org/10.1128/jcm.33.10.2592-2595.1995
8. Biju N et al (2016) High prevalence of enterocytozoon hepatopenaei in shrimps penaeus monodon and litopenaeus vannamei sampled from slow growth ponds in India. Dis Aquat Org 120(3):225–230. https://doi.org/10.3354/dao03036
9. Sudhakaran R et al (2009) A simple non-enzymatic method for the preparation of white spot syndrome virus (WSSV) DNA from the haemolymph of Marsupenaeus japonicus using FTA matrix cards. J Fish Dis 32(7): 611–617. https://doi.org/10.1111/j.1365-2761.2009.01042.x
10. Karthikeyan K, Saranya R, Bharath R, Vidya R, Itami T, Sudhakaran R (2020) A simple filter paper-based method for transporting and storing Enterocytozoon hepatopenaei DNA from infected Litopenaeus vannamei tissues. J Invertebr Pathol 169(December 2019):107305. https://doi.org/10.1016/j.jip.2019.107305
11. Karthikeyan K, Sudhakaran R (Mar. 2019) Experimental horizontal transmission of Enterocytozoon hepatopenaei in post-larvae of whiteleg shrimp, Litopenaeus vannamei. J Fish Dis 42(3):397–404. https://doi.org/10.1111/JFD.12945
12. Wang L et al (2020) Integrated qpcr and staining methods for detection and quantification of enterocytozoon hepatopenaei in shrimp litopenaeus vannamei. Microorganisms 8(9):1–11. https://doi.org/10.3390/microorganisms8091366
13. Gao W et al (2020) A double staining method using calcofluor white and acridine orange to differentiate life stages of Enterocytozoon hepatopenaei (EHP) on hepatopancreatic sections. Aquaculture 528:735628. https://doi.org/10.1016/j.aquaculture.2020.735628

Part II

Isolation and Identification of Probiotic Bacteria in Aquaculture

Chapter 13

Methods for Isolating and Identifying Probiotic Bacteria from Fishes

Mahalakshmi S. Patil, Raghu Ram Achar, and Ann Catherine Archer

Abstract

The growing industry of aquaculture is often faced with challenges of increased production leading to several bacterial and viral diseases in the fish. Antibiotics and chemicals are commonly used to prevent and control diseases. However, injudicious use of antibiotics has led to a serious threat of antibiotic resistance spread in the fish consequently resulting in the transfer of resistance genes from pathogens to other microflora. To keep up with the increasing demand of the food industry and safeguard the health of humans, fish, and the environment, probiotics are considered as a safer and environment-friendly alternative. As marine and freshwater fish species share an intricate relationship with their environment, the microbiota of fish differ from species to species and thus require robust probiotic strains to target specific species and disease pathogens. Isolating probiotic bacteria from the host environment is ideal as they possess the abilities to adapt and colonize in the host and confer beneficial effects. This chapter outlines the methods for the isolation of potential probiotic microorganisms and techniques used for their identification.

Key words Probiotics, Probiotic bacteria, Lactic acid bacteria, Aquaculture, Fingerprinting, RAPD, 16S rRNA gene sequencing

1 Introduction

1.1 Probiotics and Probiotic Bacteria

Probiotics are defined as "live microorganisms when consumed in adequate amounts confer a health benefit on the host" [1]. The term "probiotic" meaning "for life" is derived from the Greek words "pro" and "bios." The concept of probiotic was first proposed by Élie Metchnikoff when he observed that consumption of fermented milk reduced putrefactive microorganisms and promoted health and well-being. Nowadays, probiotics are used as prophylactic, therapeutic, and growth supplements for both human and animal health [2]. Probiotics have a beneficial effect through various mechanisms including competitive exclusion of pathogens, pathogen inhibition, production of enzymes, modulation of immune system and gut microbiota, and improving

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_13,

digestion [3, 4]. Commonly studied probiotic microorganisms include lactic acid bacteria (LAB), *Bacillus* spp., and even some species of yeast. LAB are the most widely studied bacteria as probiotics due to their long history of use in fermented foods and safety [5]. LAB are also naturally present in the gastrointestinal tract of humans, animals, and fishes. Being commensal residents of the gut, LAB have the ability to tolerate the acidic and bile conditions of the gut, reduce pH by production of lactic acid, and prevent colonization of pathogens [6].

1.2 Need for Probiotics in Fisheries

Aquaculture is a rapidly growing industry particularly in the developing nations due to the rising population, demand for healthy protein sources, and decline of fishes in inland sources [3, 7]. With the industry geared to meet the ever-increasing needs of food insecurity and malnutrition, the burden of fish farming leads to increased disease susceptibility and spoilage of catch. While healthy animal husbandry methods can reduce the disease burden, other biocontrol agents such as chemicals and antibiotics are used for prevention and disease control [8]. However, use of chemicals and antibiotics poses potential health risks for consumers and environment as they can bioaccumulate in the fish, remain as residue in tissues, and more importantly cause problem of antibiotic resistance [9–11]. As antibiotic-resistant genes can be horizontally transferred to other bacteria, it is essential to adopt measures to curb the spread of resistance and safeguard the health of fish, humans, and the environment [12]. Probiotics are being extensively investigated as safe and eco-friendly alternative to improve production, extend shelf life, and mitigate disease problems in the aquaculture sector [13–16].

1.3 Isolation of Probiotics

In the past, probiotics for aquaculture were mainly derived and adapted from human and terrestrial animals ignorant of the fact that the physiology of aquatic species is significantly different and their relation with the environment is much more intricate [17]. For aquaculture applications, probiotics are generally isolated from microbiota both indigenous and exogenous microbiota of aquatic animals. While Gram-negative bacteria like *Vibrio* and *Pseudomonas* dominate the indigenous flora of marine fish, genera *Aeromonas*, *Bacteroides*, *Fusobacterium*, and *Eubacterium* are present in freshwater species. LAB are found to be subdominant in fishes [18]. Thus, probiotic bacteria isolated from host fish of interest are likely to adapt and fare better than their terrestrial counterparts. Also, probiotic effects are strain specific. Hence, rigorous screening of indigenous isolates is essential to select a potential isolate for probiotic application. Initial screening using in vitro methods is ideal for isolating and identifying a potential strain although further tests are required to prove its beneficial effects. Apart from LAB and *Bacillus*, other genera of bacteria including

Aeromonas, *Arthrobacter*, *Clostridium*, *Bifidobacterium*, *Paenibacillus*, *Pseudomonas*, *Roseobacter*, *Rhodosporidium*, *Streptomyces*, and *Vibrio*, microalgae, and yeast are also used as probiotics for aquaculture. The methods used for the isolation of probiotic bacteria are outlined in this chapter. Since isolation is always accompanied by identification of the isolate, methods for identification are also described.

2 Materials

2.1 Media Composition

2.1.1 Lactobacillus deMan, Rogosa, and Sharpe Agar (MRS):

Ingredients	Grams/liter
Protease peptone	10.000
Peptone bacteriological	10.000
Yeast extract powder	5.000
Dextrose (Glucose)	20.000
Polysorbate 80 (Tween 80)	1.000
Ammonium citrate	2.000
Sodium acetate	5.000
Magnesium sulfate	0.100
Manganese sulfate	0.050
Dipotassium hydrogen phosphate	2.000
Agar	12.000
Final pH (at 25 °C)	6.5 ± 0.2

2.1.2 Tryptic Soy Agar

Ingredients	Grams/liter
Tryptone	17.000
Soy peptone	3.000
Sodium chloride	5.000
Dextrose (glucose)	2.500
Dipotassium hydrogen phosphate	2.500
Agar	15.000
Final pH (at 25 °C)	7.3 ± 0.2

2.1.3 Nutrient Agar

Ingredients	Grams/liter
Peptone	5.000
Sodium chloride	5.000
HM peptone B#	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25 °C)	7.4 ± 0.2

2.1.4 Miller Luria Bertani Broth

Ingredients	Grams/liter
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25 °C)	7.5 ± 0.2

2.1.5 Zobell Marine Agar

Ingredients	Grams/liter
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulfate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium chloride	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000
Final pH (at 25 °C)	7.6 ± 0.2

2.2 Fine Chemicals

Saline (0.85%), phosphate buffer solution (PBS pH—7.4), peptone water (1%), glycerol, ethanol (70%), ultrapure water, chloride (10%), clove oil, ethyl alcohol, 2-phenoxyethanol, ethyl p-aminobenzoate, tricaine methane sulfonate, bromocresol purple, sodium chloride (NaCl), benzalkonium chloride (0.1%), hydrogen peroxide, crystal violet, Gram's iodine, Gram's decolorizer, safranin.

2.3 Molecular Biology Reagents

Tris–EDTA buffer (TE buffer), lysozyme, proteinase K, sodium dodecyl sulfate (SDS), Tris-saturated phenol, chloroform, sodium acetate, absolute ethanol, agarose, Tris–acetic acid–EDTA (TAE) buffer, ethidium bromide, 6× gel loading dye, 10 Kb DNA ladder, PCR buffer, $MgCl_2$, dNTPs, *Taq* DNA polymerase, nuclease-free water.

3 Methodology

3.1 Sample Collection

1. Harvest fishes from freshwater (streams, rivers, ponds, and lakes) and marine water (oceans and seas) by professional fisheries through netting, dredging, trawl netting, drift netting, harpooning, basket traps, hooks and baits, dip net, and seine net [19, 20].
   - Fish sample include freshwater and marine water species, namely, *Tilapia species (second most farmed fish species worldwide), anchovy fish (dried and salted), grouper and loach, shellfish samples (prawn, shrimp, crap, conch), mullet, carp, white bream, goby, rainbow trout, Sheedal, sea bass, red paree, African nightcrawler, Budu (fermented fish), Channa striata, Puntius filamentous, Oreochromis mossambicus, Rasbora daniconius, Ballan wrasse, white seabream, Peter's fish, European conger, red scorpionfish, gray mullet, zebra sea bream, longfin gurnard, dream fish, small-spotted catshark, triggerfish, cuckoo wrasse, carp rohu, Labeo rohita, wild-caught mullets (Mugil cephalus, Chelon ramada, Chelon labrosus, Chelon saliens), barramundi fish, Scorpis violacea, Barbonymus gonionotus, Girella melanichthys, and Cyprinus carpio.*
2. The harvested fish are pre-treated to remove surface contamination by either of the two methods mentioned below:

   Method 1

   1. Collect the fish from freshwater and marine water and subject to surface (body) wash by benzalkonium chloride (0.1% for 1 min), followed by anesthetization using ethyl-p-aminobenzoate (0.04 g), clove oil, and ethyl alcohol diluted in water (to reduce concentration) or by dipping in tricaine methanesulfonate/sharp blow on fish head and isolation is carried out [21–23].

Method 2

1. Introduce the fish into indoor acclimatization with regular maintenance and feed plant-based or basal diet (composition—fish meal, soybean meal, ground corn, wheat flower, vegetable oil, cod liver oil, dicalcium phosphate, gluten, corn starch, canola oil, carboxymethyl cellulose, α-starch, mineral mix, vitamin mix) in the form of pellet twice daily and keep under observation.
2. Prior to isolation, conduct a starvation period for 48 h and kill either by injecting or dipping in above mentioned organic solvents [24, 25].

3.2 Isolation

3.2.1 Serial Dilution and Plating (Fig. 1) [26–29]

1. Sacrifice the fish and dissect aseptically with sterile scissors/tweezers required parts such as the stomach, liver, kidney, gills, skin, gonads, and gut. Homogenize in sterile saline/PBS buffer (pH 7.4)/1% peptone water. Prominently, fish gut (intestine) is preferred for isolation.
2. Prepare test tubes containing 9 mL of saline/PBS/peptone water with cotton plugs and autoclave at 121 °C for 20 min.
3. Add 1 mL of the homogenized sample to the first tube labeled 10^{-1}. Mix well and transfer 1 mL of aliquot from the first tube to the second tube labeled 10^{-2}.

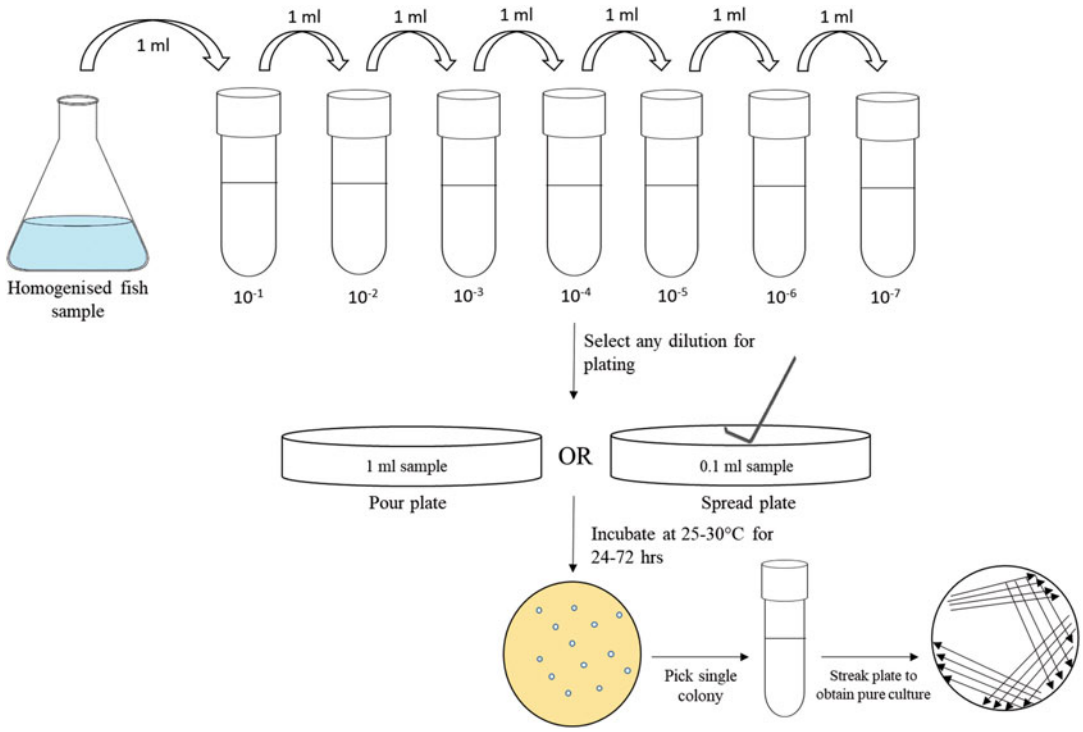

Fig. 1 Serial dilution and plating method for isolation of probiotic bacteria

4. Repeat the dilution serially to subsequent tubes up to 10^{-7} or 10^{-9} or as desired.
5. Add 0.1 mL aliquot from any of the desired dilution and pour plate on MRS/TSA/NA/LB/ZMA agar. The agar medium is chosen based on the species you wish to isolate.
6. Agar media can be supplemented with indicators such as bromocresol purple or sodium chloride (1–2%). Bromocresol purple is used to indicate colonies of LAB. Sodium chloride is added to marine fish samples to mimic the marine water conditions.
7. Incubate the petri plates at 25–30 °C for 24–72 h and observe the growth of colonies, and calculate the colony-forming units using the following formula:

$$\text{CFU/ML} = \frac{\text{Total no.of colonies} \times \text{dilution factor}}{\text{Volume of sample plated}}$$

Observation: Pinpoint colonies with different colony characteristics can be selected, picked, and cultured in preferred liquid media for further characterization.

Plates containing bromocresol purple will show yellow coloration around the colonies due to acid production indicative of LAB.

3.2.2 Purification of Isolates

1. Overnight grown cultures in broth are streaked onto MRS/TSA/NA/LB agar plates to obtain isolated purified cultures.
2. Pick single colony from the streak plate and culture in respective broth.
3. Prepare glycerol stock by adding pure culture with 40% glycerol in 1:1 ratio in an Eppendorf tube and store at −20 °C.

3.3 Preliminary Identification Tests [30]

1. Gram's staining: Perform Gram's staining to determine whether the isolate is Gram positive or Gram negative.
   (a) Briefly, smear a loopful of culture on a clean slide and heat fix. Flood the smear with primary stain crystal violet and keep for 1 min.
   (b) Wash with tap water. Add Gram's iodine for 1 min followed by decolorizer for 30 s. Finally, stain with safranin (counterstain) for 45 s.
   (c) Wash the stain off with water, dry the slide, and observe under the microscope.
   (d) Cells appearing blue or purple in color are Gram positive, while cells stained pink are Gram negative.
   (e) Morphology of the cells (cocci/bacilli/streptococci/coccobacillus, etc.) is recorded.

2. Catalase test: Catalase test is performed to test the ability of the isolate to break down hydrogen peroxide by producing catalase enzyme.
   (a) Add few drops of hydrogen peroxide (H_2O_2) to a loopful of culture on a clean glass slide.
   (b) Release of effervescence indicates catalase-positive reaction, while no effervescence is catalase-negative.

   Note: LAB are Gram-positive and catalase-negative organisms, while *Bacillus* spp. are Gram-positive and catalase-positive.

3.4 Phenotypic Characterization [31–34]

Bacterial isolates are grown at different physiological conditions such as temperature, pH, and salt concentrations and in the presence of different sugars for preliminary identification of the isolates to the genera and species level.

3.4.1 Growth of Bacterial Isolates at Different Temperatures

1. Freshly prepare MRS/TS/LB broth in a test tube (5 mL) with cotton plug and sterilize.
2. Inoculate 1% overnight grown cultures in test tubes and place them at different temperature for 24–48 h to observe the growth.
3. Observation: The growth is represented as follows:
   1. +/- = indicates no growth
   2. + = indicates growth
   3. ++ = indicates good growth
   4. +++ = indicates luxuriant growth

   (applicable for all physiological and biochemical tests)

3.4.2 Growth of Bacterial Isolates at Different pH

1. Freshly prepare MRS/TS/LB medium and adjust the pH to either acidic or basic using pH meter.
2. For broth of acidic pH, adjust the broth to acidic pH 2 and 3 by adding concentrated HCl, and handle by wearing gloves, eye protector, and mask.
3. To prepare broth of basic pH, dilute NaOH pellets in water and then add the dilute solvent dropwise to bring the broth to basic pH 7 and 9.
4. Inoculate 1% of overnight grown culture in pH (acidic or basic) broth and incubate for 24–48 h at 25–30 °C in an incubator. Note down the results thereafter.

3.4.3 Growth of Bacterial Isolates at Different Salt Concentrations

1. Supplement the MRS with different salt concentrations and keep for sterilization.
2. Subculture the isolates and incubate at 37 °C for 24 h.

3. Inoculate 50 μL of culture in sterilized test tubes containing broth with addition of NaCl at different concentrations (can range between 4% and 6.5% depending on the organism of interest) and incubate for 24 h at 25–30 °C in an incubator and results are to be noted.

3.4.4 Sugar Fermentation Test

This test is mainly carried out for LAB isolates as they show varied utilization of sugars depending on their fermentation abilities.

1. Supplement MRS broth with different sugars (1%) and phenol red indicator. Place Durham's tube in an inverted position in the test tube and sterilize.
2. Inoculate 1% of overnight grown culture into the sterile broth and incubate for 24–48 h at 25–30 °C.
3. Observation: The isolates are differentiated as homofermentative (acid production—change in color from red to yellow) or heterofermentative (acid and gas production—change in color from red to yellow and gas bubble formation in Durham's tube).
4. The results of all the tests will be tabulated as given in Table 1.

3.5 Molecular Identification

Physiological and biochemical tests help in tentative identification of the isolates. However, their accurate identification at the species level requires the use of molecular techniques. DNA is isolated from the pure cultures of bacterial isolates which is subsequently used as template for subsequent identification by PCR (polymerase chain reaction) methods.

3.5.1 Total DNA Isolation [35]

Phenol/chloroform method is in practice to extract genomic DNA from bacterial suspension.

1. Centrifuge overnight grown culture (1 mL) at 8000 rpm for 10 min at 4 °C.
2. Pellet and supernatant separation are found after centrifugation.
3. Discard the supernatant and suspend the pellet by adding 500 μL of 1× TE buffer and 30 μL of lysozyme, recommended to mix thoroughly, and incubate in water bath for 1 h at 37 °C.

Table 1
Physiological tests of the isolates

Sl. no	Isolates	Temperature (°C)	pH	Salt concentrations	Fermentation test
01	Isolate-1				
02	Isolate-2				
03	Isolate-3				

4. Add 15 µL of proteinase K and SDS (20%) gently to get a clear solution and incubate in water bath for 30 min at 55 °C.
5. Add Tris-saturated phenol (250 µL) and centrifuge at 8000 rpm for 15 min at 4 °C.
6. Observe phase separation at this step and collect upper aqueous phase carefully without dragging the sediment.
7. After phase separation, mix chloroform (400 µL) to upper aqueous phase and centrifuge at 10,000 rpm for 15 min at 4 °C.
8. Carefully collect the aqueous phase and mix it with 15 µL of sodium acetate (3 M) in addition to 400 µL of cold absolute alcohol and incubate for DNA precipitation.
9. Overnight incubate the DNA sample at 4 °C and centrifuge the next day at 10,000 rpm for 10 min at 4 °C.
10. Discard the supernatant and wash the pellet by adding 70% absolute alcohol (50 µL) and centrifuge at 10,000 rpm for 10 min at 4 °C.
11. Obtain the pellet and air-dry for 5–10 min at 30–37 °C, suspend the pellet in 50 µL of nuclease-free water or TE buffer and store at -20 °C.
12. Quality of DNA is checked by agarose gel electrophoresis.

3.5.2 Agarose Gel Electrophoresis

1. Dissolve 0.8% of agarose in Tris–acetic acid (TAE buffer) and homogenize in microwave oven, and allow to cool at 50 °C.
2. Pour the agarose into the casting tray, seal with adhesive tape, and place the comb to make wells.
3. Place the casting tray to electrophoresis tank containing 1× TAE buffer.
4. Load DNA sample (4 µL) with 2 µL of 6× gel loading (tracking) dye.
5. Run the gel at 80 V till tracking dye reaches to three fourths of the gel.
6. Stain the gel by immersing in a container containing ethidium bromide solution.
7. Visualize the bands in a UV transilluminator.
8. Band observed below the well of the gel indicates the presence of DNA. Thick and intact bands indicate quality and quantity of the DNA obtained.
9. Document the results in a gel documentation system or by taking images under UV illumination.

3.5.3 Random Amplified Polymorphic DNA (RAPD-PCR)

RAPD is a PCR-based technique used to differentiate between species and strains. It is generally used as a fingerprinting tool in identification of isolates. Universal primers such as M13 can be used for RAPD PCR [36].

1. Prepare a PCR reaction mixture (25 μL) consisting of 50 ng/μL of DNA as template, 10 pmol of forward and reverse primer each, 10× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 U *Taq* DNA polymerase.
2. Add the reaction mixture to 0.2 mL PCR tubes, mix well, and spin down the samples in a microcentrifuge.
3. Place the tubes in a thermal cycler and set the program as follows: initial denaturation at 95 °C for 5 min followed by 35–40 cycles of denaturation at 95 °C for 1 min, annealing at appropriate temperature (to be decided based on the primer chosen) for 1 min, and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min.
4. Observe the amplified product by agarose gel electrophoresis using 1.8% agarose gel. Run the samples alongside a 10 Kb DNA ladder. Document the results.
5. Observe the presence of multiple bands in each lane and compare between isolates for similar or different banding patterns (Fig. 2a).

3.5.4 16S rRNA Gene Sequencing [37]

16S rRNA gene sequencing is in practice to identify the unidentified bacteria at a species level and genus level and differentiate between closely related bacterial species. Amplification of 16S rRNA gene is done by using universal primers: 27F and 1492R.

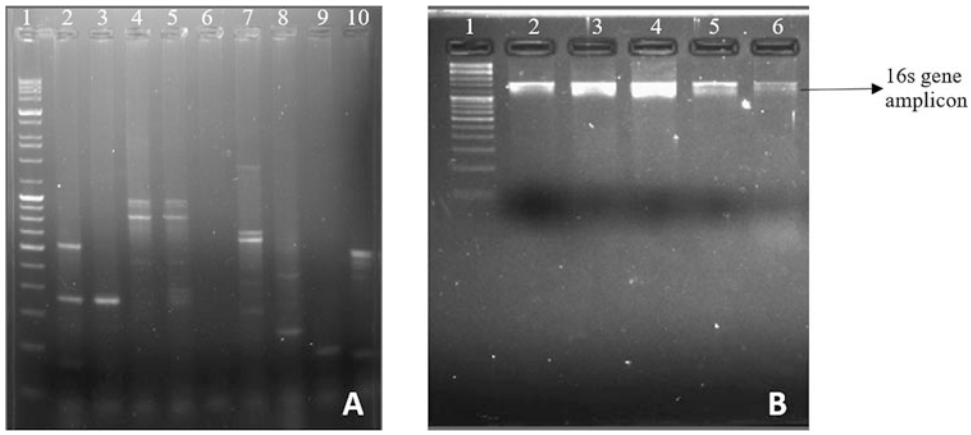

Fig. 2 Agarose gel image of RAPD PCR of LAB isolates (**a**). Lane 1 is DNA ladder, and Lanes 2–9 are banding patterns (fingerprint) obtained from amplified DNA samples of LAB isolates. (**b**) 16s rRNA gene PCR of LAB isolates. The amplicon size is 1500 bp corresponding to the DNA ladder (*Unpublished data of Ann Catherine Archer*)

1. Prepare the PCR reaction mixture as mentioned in Subheading 3.5.3 with 16S primers. Load the samples in a thermal cycler with the same program as mentioned in Subheading 3.5.3. The annealing temperature is fixed based on the choice of primer.
2. Run the amplified products on 1% agarose gel at 80 V and visualize the band corresponding to the expected product size of the amplicon (Fig. 2b).
3. Excise the band and send it for sequencing. Subject the sequence to BLAST search (https://blast.ncbi.nlm.nih.gov). Hits of 98–100% identity are considered for species identification.

Acknowledgments

The authors wish to express their sincere gratitude to the authorities at JSS Academy of Higher Education & Research, Mysuru, for extending necessary support and facilities. ACA gratefully acknowledges the DBT Wellcome Trust India Alliance for grant of Early Career Fellowship (IA/E/20/1/505689). MSP acknowledges the support of Karnataka Govt. for OBC fellowship (OBC-10/PhD/CR-04/2021-22).

References

1. FAO/WHO (2001) Report of a joint FAO/-WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Córdoba, Argentina
2. Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. *Aquaculture 274*(1):1–14
3. El-Saadony MT, Alagawany M, Patra AK, Kar I, Tiwari R, Dawood MA, Dhama K, Abdel-Latif HM (2021) The functionality of probiotics in aquaculture: an overview. *Fish Shellfish Immunol 117*:36–52
4. Archer AC, Halami PM (2015) Probiotic attributes of Lactobacillus fermentum isolated from human feces and dairy products. *Appl Microbiol Biotechnol 99*(19):8113–8123
5. Archer AC, Muthukumar SP, Halami PM (2015) Anti-inflammatory potential of probiotic Lactobacillus spp. on carrageenan induced paw edema in Wistar rats. *Int J Biol Macromol 81*:530–537
6. Ringø E, Van Doan H, Lee SH, Soltani M, Hoseinifar SH, Harikrishnan R, Song SK (2020) Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. *J Appl Microbiol 129*(1):116–136
7. Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, Little D, Ross L, Handisyde N, Gatward I, Corner R (2010) Aquaculture: global status and trends. *Philos Trans R Soc B 365*(1554):2897–2912
8. Abdel-Latif HM, Dawood MA, Menanteau-Ledouble S, El-Matbouli M (2020) The nature and consequences of co-infections in tilapia: A review. *J Fish Dis 43*(6):651–664
9. Dadar M, Dhama K, Vakharia VN, Hoseinifar SH, Karthik K, Tiwari R, Khandia R, Munjal A, Salgado-Miranda C, Joshi SK (2017) Advances in aquaculture vaccines against fish pathogens: global status and current trends. *Rev Fish Sci Aquac 25*(3):184–217
10. Assefa A, Abunna F (2018) Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. Vet Med Int 2018: 5432497
11. Pérez-Sánchez T, Mora-Sánchez B, Balcázar JL (2018) Biological approaches for disease control in aquaculture: advantages, limitations and challenges. *Trends Microbiol 26*(11):896–903

12. Santos L, Ramos F (2018) Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. *Int J Antimicrob Agents 52*(2):135–143
13. Amoah K, Huang QC, Tan BP, Zhang S, Chi SY, Yang QH, Liu HY, Dong XH (2019) Dietary supplementation of probiotic Bacillus coagulans ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, Litopenaeus vannamei. *Fish Shellfish Immunol 87*:796–808
14. Azad MAK, Islam SS, Sithi IN, Ghosh AK, Banu GR, Bir J, Huq KA (2019) Effect of probiotics on immune competence of giant freshwater prawn Macrobrachium rosenbergii. *Aquacult Res 50*(2):644–657
15. Balcázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Muzquiz JL, Girones O (2008) Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture 278*(1-4):188–191
16. Araujo C, Munoz-Atienza E, Poeta P, Igrejas G, Hernandez PE, Herranz C, Cintas LM (2016) Characterization of Pediococcus acidilactici strains isolated from rainbow trout (Oncorhynchus mykiss) feed and larvae: safety, DNA fingerprinting, and bacteriocinogenicity. *Dis Aquat Organ 119*(2):129–143
17. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev 64*(4):655–671
18. Balcázar JL, De Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL (2006) The role of probiotics in aquaculture. *Vet Microbiol 114*(3-4):173–186
19. Patel P, Patel B, Amaresan N, Joshi B, Shah R, Krishnamurthy R (2020) Isolation and characterization of *Lactococcus garvieae* from the fish gut for *in vitro* fermentation with carbohydrates from agro-industrial waste. Biotechnol Rep (Amst) 28:e00555
20. Tanaka D, Ohnishi KI, Watanabe S, Suzuki S (2021) Isolation of cellulase-producing Microbulbifer sp. from marine teleost blackfish (Girella melanichthys) intestine and the enzyme characterization. J Gen Appl Microbiol 67(2): 47–53
21. Makridis P, Kokou F, Bournakas C, Papandroulakis N, Sarropoulou E (2021) Isolation of *Phaeobacter* sp. from Larvae of Atlantic Bonito (*Sarda sarda*) in a Mesocosmos Unit, and Its Use for the Rearing of European Seabass Larvae (*Dicentrarchus labrax* L.). Microorganisms 9(1):128
22. Valderrama B, Ruiz JJ, Gutiérrez MS, Alveal K, Caruffo M, Oliva M, Flores H, Silva A, Toro M, Reyes-Jara A, Navarrete P (2021) Cultivable Yeast Microbiota from the Marine Fish Species *Genypterus chilensis* and *Seriolella violacea*. J Fungi (Basel) 7(7):515
23. Sharifuzzaman SM, Rahman H, Austin DA, Austin B (2018) Properties of Probiotics Kocuria SM1 and Rhodococcus SM2 Isolated from Fish Guts. Probiotics Antimicrob Proteins 10(3):534–542
24. Abomughaid MM (2020) Isolation and Identification of Some Probiotic Bacteria and Their Potential Role in Improving Immune Response and Resistance of Nile Tilapia *(Oreochromis niloticus*) in Comparison with a Commercial Product. Int J Microbiol 2020: 8865456
25. AlKalbani NS, Turner MS, Ayyash MM (2019) Isolation, identification, and potential probiotic characterization of isolated lactic acid bacteria and in vitro investigation of the cytotoxicity, antioxidant, and antidiabetic activities in fermented sausage. Microb Cell Fact 18(1):188
26. do Vale Pereira G, da Cunha DG, Pedreira Mourino JL, Rodiles A, Jaramillo-Torres A, Merrifield DL (2017) Characterization of microbiota in Arapaima gigas intestine and isolation of potential probiotic bacteria. J Appl Microbiol 123(5):1298–1311
27. Alonso S, Carmen Castro M, Berdasco M, de la Banda IG, Moreno-Ventas X, de Rojas AH (2019) Isolation and Partial Characterization of Lactic Acid Bacteria from the Gut Microbiota of Marine Fishes for Potential Application as Probiotics in Aquaculture. Probiotics Antimicrob Proteins 11(2):569–579
28. Wanka KM, Damerau T, Costas B, Krueger A, Schulz C, Wuertz S (2018) Isolation and characterization of native probiotics for fish farming. BMC Microbiol 18(1):119
29. Samson JS, Choresca CH Jr, Quiazon KMA (2020) Selection and screening of bacteria from African nightcrawler, Eudrilus eugeniae (Kinberg, 1867) as potential probiotics in aquaculture. World J Microbiol Biotechnol 36(1):16
30. El-Jeni R, El Bour M, Calo-Mata P, Böhme K, Fernández-No IC, Barros-Velázquez J, Bouhaouala-Zahar B (2016) In vitro probiotic profiling of novel Enterococcus faecium and Leuconostoc mesenteroides from Tunisian freshwater fishes. Can J Microbiol 62(1):60–71
31. Pang Sing T, Julian R, Hatai K (2019) Identification, growth profile and probiotic properties of autochthonous intestinal bacteria of sagor

catfish (Hexanematichthys sagor). Biocontrol Sci 24(1):1–11

32. Masduki F, Jasmin MY, Min CC, Karim M (2020) Characterization of Enterococcus hirae Isolated from the Intestine of Seabass (Lates Calcarifer) as a New Potential Probiotic against Pathogenic Vibrios. Curr Microbiol 77(12):3962–3968
33. Majumdar RK, Gupta S (2020) Isolation, identification and characterization of Staphylococcus sp. from Indian ethnic fermented fish product. Lett Appl Microbiol 71(4):359–368
34. Mortezaei F, Royan M, Allaf Noveirian H, Babakhani A, Alaie Kordghashlaghi H, Balcázar JL (2020) In vitro assessment of potential probiotic characteristics of indigenous Lactococcus lactis and Weissella oryzae isolates from rainbow trout (Oncorhynchus mykiss Walbaum). J Appl Microbiol 129(4):1004–1019
35. Mora D, Fortina MG, Parini C, Daffonchio D, Manachini PL (2000) Genomic subpopulations within the species Pediococcus acidilactici detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains. Microbiology 146(8):2027–2038
36. Schillinger U, Yousif NM, Sesar L, Franz CM (2003) Use of group specific and RAPD-PCR analyses for rapid differentiation of Lactobacillus strains from probiotic yogurts. Curr Microbiol 47(6):453–456
37. Liu XF, Li Y, Li JR, Cai LY, Li XX, Chen JR, Lyu SX (2015) Isolation and characterisation of Bacillus spp. antagonistic to Vibrio parahaemolyticus for use as probiotics in aquaculture. World J Microbiol Biotechnol 31(5):795–803

Chapter 14

Isolation of Probiotic Bacteria from Gut of the Aquatic Animals

S. Vidhya Hindu and John Thomas

Abstract

Intestinal probiotic bacteria have a constant and dynamic impact on the gut and systemic immune systems of the host when taken in adequate proportions. When probiotic microorganisms are provided in functional food, they can provide health advantages. Probiotics may provide health benefits such as immune system modulation and antibacterial, anticancer, and antimutagenic properties. Probiotic bacteria can be isolated and characterized from the intestines of aquatic animals. In this chapter, we addressed the procedure of isolation, identification, and antagonistic effect of intestinal bacteria as a probiotic effect.

Key words Probiotic bacteria, Antibiotic, Aquaculture, Gut microbiota, Aquatic animals

1 Introduction

Aquaculture has been growing for decades, despite the fact that outbreaks of a number of illnesses have resulted in economic losses [1]. Aquaculture's contribution to animal protein production has skyrocketed, and it now accounts for about half of all fish and shellfish consumed globally, constituting it a major food producer [2]. Disease outbreaks, particularly viral and bacterial disease, have had a substantial impact on the aquaculture business in recent years. Chemical and antibiotic medications are extremely hazardous, and they can lead to pathogenic microorganisms that are resistant to antibiotics [3]. In order to cause disease, many pathogenic bacteria require adhesion to the mucosal layer of the human gastrointestinal tract. Competition for attachment sites, often known as "competitive exclusion," is a key mode of action in probiotic bacteria. A desirable factor in the selection of probiotics is the ability of bacteria to colonize the gut and stick to the epithelial surface, hence interfering with pathogen adherence [4].

Probiotics are beneficial microorganisms that are being considered as an effective and environmentally friendly alternative to

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_14,

antibiotics [5]. Probiotics can be a good choice for increasing aquaculture productivity in a long-term sustainable manner. For the desired advantages of probiotics application, it is vital to choose the proper strain and dose for each aquaculture species [6]. Probiotics are live beneficial bacteria which are introduced into the gastrointestinal tract via food or water, improving the internal microbial balance and promoting overall health [7]. In aquaculture, bacteria derived from the intestines of both aquatic and terrestrial animals are routinely employed as probiotics [8]. Bacteriocins, hydrogen peroxide, siderophores, lysozymes, and proteases are just a few substances produced by probiotic bacteria that have bactericidal or bacteriostatic effects on other microbial populations [9–11].

2 Materials

2.1 Collection of Sample

- Aquatic animals.
- Aeration.
- Tank.
- Thermometer.
- pH pen.
- Weighing balance.
- Commercial feed for aquatic animals.

2.2 Isolation of Intestinal Bacteria

- Dissection box.
- Tricaine methanesulfonate (MS-222).
- Normal sterile saline (NaCl 0.85% w/v).
- Laminar airflow.
- Mortar and pestle.
- Tryptic soy agar.
- L-rod.
- Incubator.
- Refrigerator.
- Pipettes.

2.3 Antagonistic Effect of Isolated Bacteria

- Pathogenic bacteria.
- Isolated strains from the intestine.
- Petri plate.
- Calorimeter.
- Mueller Hinton agar plate.

- Swab stick.
- Pipettes.
- Incubator.
- Laminar airflow.
- Incubator.
- Refrigerator.

2.4 Identification of Probiotic Bacteria

- Refer to standard microbiology laboratory manual to check the morphological and biochemical characteristics.
- Bergey's Manual of Systematic Bacteriology.

2.4.1 Morphological and Biochemical Characteristics

2.4.2 Molecular Identification

- 16S ribosomal RNA (rRNA) sequence analysis.

3 Methods

3.1 Collection of Sample

- Collect the healthy aquatic animals like fish and prawn in live condition.
- Transfer the collected animals in live condition to the laboratory.
- Acclimatize the experimental animals under aerated condition for 15 days at optimum temperature prior to experiment.
- Feed the experimental animals twice a day with commercial feed.

3.2 Isolation of Intestinal Bacteria [12]

- Euthanize the three healthy animals by immersion in tricaine methanesulfonate (MS-222).
- Dissect out the intestine of animals under aseptic condition and wash thrice with normal saline (NaCl 0.85% w/v).
- Homogenize the intestine in surface-sterilized mortar and pestle using 5 mL of sterile saline (NaCl 0.85% w/v).
- Collect the homogenate sample and perform serial dilution (tenfold) using sterile saline solution.
- Transfer 0.1 mL of diluted samples from each dilution to the tryptic soy agar plate and spread evenly over agar plate using L-rod.
- Incubate the plates at 37 °C for 48 h.
- After 48 h, observe the colonies on the plate and store the plates in the refrigerator at 4 °C until further use.

3.3 Antagonistic Effect of Isolated Bacteria [12]

- Prepare the Mueller Hinton agar plates.
- Take pathogenic bacteria of your interest.
- Swab 100 μL of pathogenic strains (108 CFU/mL) over the Mueller Hinton agar plates.
- Make a well of 7 mm diameter over the agar surface.
- Prepare the cell-free supernatant by centrifuging the different isolated strains at 8000 rpm for 10 min.
- Fill the well with 50 μL of cell-free supernatants of different isolated strains.
- Allow the strains in the well to get absorb completely.
- Then incubate the plates at 37 °C for 48 h in inverted position.
- After 48 h, observe the zone of inhibition of different isolated strains against pathogenic bacteria which were swabbed over the agar plate.
- Select the strains which show strong inhibition zone against pathogen for further analysis.
- The strain which shows inhibition zone against pathogenic bacteria has the probiotic effect.

3.4 Identification of Probiotic Bacteria

3.4.1 Morphological and Biochemical Characteristic [13]

- Perform all the preliminary characterization such as staining techniques and biochemical tests using standard procedures for the isolated strains showing probiotic effect.
- Interfere the results using Bergey's Manual of Systematic Bacteriology, Eighth Edition, to conform the genus of your strain.

3.4.2 Molecular Identification

- Confirm the genus of the isolated strain using 16S rRNA sequencing.

References

1. Martinez Cruz P, Ibanez AL, Monroy Hermosillo OA, Ramirez Saad HC (2012) Use of probiotics in aquaculture. ISRN Microbiol 2012:1–14
2. Jahangiri L, Esteban MÁ (2018) Administration of probiotics in the water in finfish aquaculture systems: a review. Aust Fish 3:33
3. Zhu F (2020) A review on the application of herbal medicines in the disease control of aquatic animals. Aquaculture 526:735422
4. Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, Gomez-Llorente C, Gil A (2012) Probiotic mechanisms of action. Ann Nutr Metab 61:160–174
5. Zorriehzahra MJ, Delshad ST, Adel M, Tiwari R, Karthik K, Dhama K, Lazado CC (2016) Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. Vet Q 36:228–241
6. Subedi B, Shrestha A (2020) A review: application of probiotics in aquaculture. Int J For Anim Fish Res 4:52–60
7. El-Saadony MT, Alagawany M, Patra AK, Kar I, Tiwari R, Dawood MA, Dhama K, Abdel-Latif HM (2021) The functionality of probiotics in aquaculture: an overview. Fish Shellfish Immunol 117:36–52

8. Van Hai N, Fotedar R (2010) A review of probiotics in shrimp aquaculture. J Appl Aquac 22:251–266
9. Servin A (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. Microbiol Rev 28:405–440
10. Panigrahi A, Azad IS (2007) Microbial intervention for better fish health in aquaculture: the Indian scenario. Fish Physiol Biochem 33: 429–440
11. Tinh NTN, Dierckens K, Sorgeloos P, Bossier P (2007) A review of the functionality of probiotics in the larviculture food chain. Mar Biotechnol 10:1–12
12. Vidhya Hindu S, Chandrasekaran N, Mukherjee A, Thomas J (2018) Effect of dietary supplementation of novel probiotic bacteria *Bacillus vireti* 01 on antioxidant defence system of freshwater prawn challenged with *Pseudomonas aeruginosa*. Probiotics Antimicro Prot 10:356–366
13. Bergey's manual of systematic bacteriology (1974) 8th edn. Springer, Cham

Chapter 15

Characterization of Probiotic Properties of Isolated Bacteria

Mahalakshmi S. Patil, Raghu Ram Achar, and Ann Catherine Archer

Abstract

The growing needs of the aquaculture industry demand measures to safely increase food production by controlling the emergence and spread of pathogenic fish diseases which lead to huge economic losses. Probiotics are an alternative approach to curb the use of harmful chemical agents and antibiotics that jeopardize host and environmental health. However, by definition, a probiotic organism must be a viable strain that beneficially affects the host health, and hence microorganisms screened for probiotic applications must possess essential probiotic properties. The Food and Drug Administration (FDA) and World Health Organization (WHO) have laid out certain guidelines and criteria for the characterization of probiotic organisms. This chapter deals with some of the essential criteria used to study potential probiotic microorganisms in vitro and the methods followed to evaluate them.

Key words Probiotic bacteria, Bile salt hydrolase, Mucus adhesion, Hydrophobicity, Auto-aggregation, Antibiotic susceptibility, Antimicrobial activity

1 Introduction

Probiotics being live microorganisms that can benefit the host's health in the right amounts are increasingly being applied as a friendly alternative to prevent and control disease in aquaculture. Probiotics have shown positive effects in aquaculture including a reduction in disease burden, improved immunity and health balance, improved growth and performance, sustenance of healthy gut function, and microbial balance [1–3]. Not all commensal microorganisms are probiotic. They must fulfill certain criteria to be termed probiotic organisms. These criteria include the following: the organism should be safe to the host, should not harbor any transmissible resistance genes, should be able to colonize and replicate within the host, should tolerate conditions of the host physiology, and must show beneficial health effect in the host system. A battery of in vitro tests is conducted to characterize the probiotic properties of the microorganism followed by in vivo safety and

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_15,

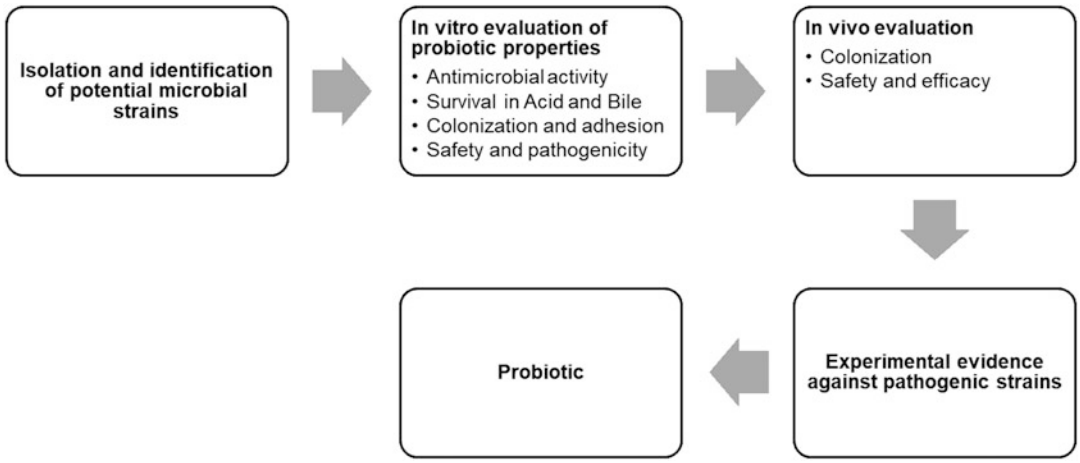

Fig. 1 General scheme of selection of probiotics for aquaculture

efficacy testing [4–6]. However, the choice of tests and methods for selection of probiotics varies in different reports.

The most common method of initial screening involves antimicrobial/antagonism tests as selecting strains that have potential antagonistic activity is the primary aim for disease prevention and control in fishes. Other parameters include blood hemolysis; antibiotic susceptibility; adherence assays like hydrophobicity, autoaggregation, and mucin adhesion; tolerance to acid and bile; etc. These tests are often preceded by collection of background information of the host and its environment, and a rich pool of potential probiotic candidates isolated preferably from the host origin [7–9]. A general scheme of probiotic selection of aquaculture is given in Fig. 1. Although some studies suggest the use of in vivo methods to screen for probiotics, this chapter will be restricted to the in vitro assays commonly used to characterize probiotic bacteria.

2 Materials

2.1 Media Preparation

The media composition required for most assays such as MRS, TSA, NA, LB, etc. is provided in the preceding chapter.

2.1.1 Mueller Hinton Agar

Ingredients	Grams/liter
Beef extract	2.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH (at 25 °C)	7.3 ± 0.1

2.1.2 Blood Agar

Ingredients	Grams/liter
Peptone	5.00
Beef extract	3.00
NaCl	5.00
Agar	15.00
Sheep blood (5%)	50 mL
Final pH (at 25 °C)	7.2 ± 0.1

2.1.3 Peptone–Gelatin Agar

Ingredients	Grams/liter
Peptone	5.00
Glucose	1.00
Gelatin	15.00
Agar	15.00
Final pH (at 25 °C)	7.3 ± 0.1

2.1.4 Starch Agar

Ingredients	Grams/liter
Peptone	5.00
Beef extract	3.00
Starch	1.00
Agar	15.00
Final pH (at 25 °C)	7.2 ± 0.1

2.1.5 Tributyrin Agar

Ingredients	Grams/liter
Peptone	5.00
Yeast extract	3.00
Agar	15.00
Tributyrin (1%)	10.00 mL
Final pH (at 25 °C)	7.5 ± 0.1

2.1.6 Carboxymethyl Cellulose Agar

Ingredients	Grams/liter
Peptone	10.00
Carboxymethyl cellulose	10.00
K_2HPO_4	2.00

(continued)

Ingredients	Grams/liter
$MgSO_4.7H_2O$	0.30
$(NH_4)_2SO_4$	2.50
Gelatin	2.00
Agar	15.00
Final pH (at 25 °C)	6.8–7.2 ± 0.1

2.2 Chemicals and Reagents

Saline, PBS, HCl, sodium chloride (NaCl), phenol red indicator, antibiotic discs, bile (Ox-gall), hydrocarbons (n-hexadecane, xylene, chloroform, toluene, n-octane, ethyl acetate), pepsin, pancreatin, taurodeoxycholic acid (TDCA), calcium chloride ($CaCl_2$), crystal violet, acetate buffer, citrate buffer, 0.1% benzalkonium chloride solution, Lugol's iodine solution, Gram's iodine, 15% $HgCl_2$.

3 Methodology

3.1 Safety Tests

Probiotic bacteria are said to be beneficial organisms, and hence one of the criteria for probiotic selection is that they should be safe and not contain any virulence factors or harbor any transmissible antibiotic resistance genes.

3.1.1 Hemolytic Activity

The fish gut harbors several pathogens such as *Aeromonas* which possess virulence genes like aerolysin and hemolysin capable of hemolyzing blood cells [10, 11]. Human blood, sheep blood, fish blood, horse blood, etc. can be used to test the hemolytic activity of the isolates [12, 13]. Probiotic bacteria unlike pathogens should not possess any hemolytic activity.

1. Prepare a rich medium like blood agar supplemented with 5% fresh sheep blood (most commonly, sheep blood is at the forefront for this activity, while few organisms need rabbit or bovine blood as the hemolytic activity depends on the type of blood chosen).
2. Culture the isolates to be tested in sterile test tubes.
3. Streak or spot a loopful of overnight grown culture onto sheep blood agar plates and incubate for 24–48 h at 37 °C [14, 15].
4. Observation: Observe the presence/absence of zone formation around the colonies to interpret the hemolysis activity.
5. Interpretation of results: Hemolytic activity representation is as follows (Fig. 2):

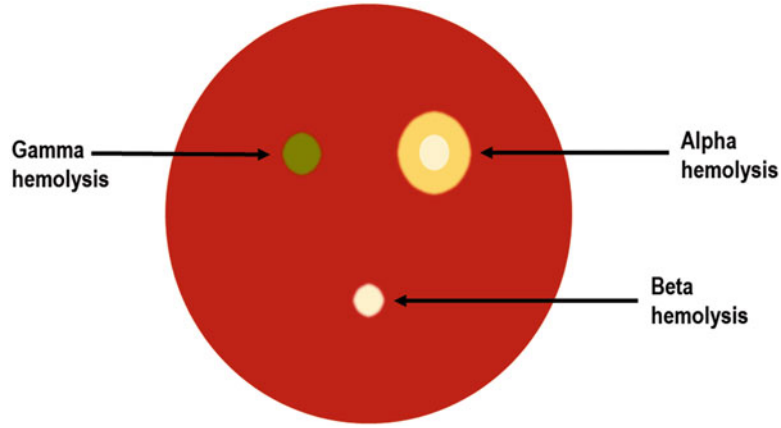

Fig. 2 Result interpretation of hemolytic activity on a blood agar plate

α-hemolysis—greenish to the brownish zone around the colony

β-hemolysis—clear zone appearance

γ-hemolysis—no zone appearance

3.1.2 Antibiotic Susceptibility Test

An antibiotic susceptibility test is done to determine the sensitivity or resistance of potential probiotic bacteria against various antibiotics. The Kirby–Bauer disc diffusion method is the standard method used to carry out this test. This method is most widely in practice, which relies on measuring inhibition of bacterial growth according to instructions set down from CLSI (Clinical and Laboratory Standards Institute) and FDA (Food and Drug Administration) [16].

1. Grow the test cultures in liquid broth in test tubes and incubate overnight.
2. Spread overnight grown cultures on Mueller Hinton agar (MHA) by swab technique (immerse sterile cotton swabs in overnight grown cultures and spread onto MHA medium).
3. Prepare appropriate concentrations of antibiotics and impregnate them onto small circular discs cut in Whatman paper. Alternatively, you can use ready-made antibiotic discs available from HiMedia Laboratories (Mumbai).
4. Place the antibiotic-impregnated discs on the agar (take care to pick up the antibiotic disc with sterile forceps, and place them 2 cm apart from each other to circumvent zone combat).
5. Gently press the discs with the forceps to ensure that they stay in place.
6. Incubate the plates for 16–18 h at 25–30 °C.

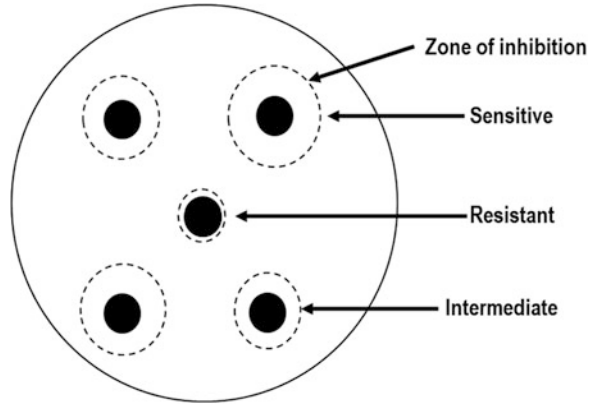

Fig. 3 Result interpretation of antibiotic susceptibility by disc diffusion method

7. Observation: Observe the formation of the inhibition zone around the discs. The diameter of the zone indicates the susceptibility of the culture to the respective antibiotic [17, 18].
8. Measure zone of inhibition in mm and compare the diameter of zones to susceptibility table with approval from the Food and Drug Administration (FDA) and Clinical and Laboratory Standards Institute (CLSI).
9. Result interpretation of disc diffusion test (Fig. 3):

   Sensitive—treatable by normal dosage

   Intermediate—may respond to the higher dosage

   Resistant—unlikely to respond to the usual dosage

3.2 Probiotic Properties

3.2.1 Antimicrobial Activity

Antimicrobial activity is an essential probiotic property for application in aquaculture as the primary aim is to prevent and control diseases in fish by inhibiting pathogens [3, 5]. Hence, this property is the first and most common method used to characterize a potential probiotic bacterium and is often also used as a test to screen for bacteria having antagonistic activity. Several methods such as spot-on lawn, double agar layer, and agar well diffusion assays are used [19, 20]. The well diffusion assay being the widely used method is described in this chapter. Other methods and further characterization of antimicrobial activity are dealt with in detail in the following chapter.

1. Inoculate test cultures in appropriate broth and incubate for 24 h. Similarly, grow pathogen cultures (indicator bacteria) in BHI broth and incubate at 37 °C for 24 h.
2. Collect the cell-free supernatant (CFS) of test cultures by centrifuging at 8000 rpm for 10 min and filter the CFS through a 0.22 μm filter.
3. Swab the indicator bacteria on Mueller Hinton or BHI agar plates.

4. Bore wells in each plate and add the CFS in the respectively labeled wells. Incubate the plates at 37 °C for 24 h.
5. Observe for a clear zone of inhibition around the wells and measure the diameter of the zones to determine the inhibitory activity [21, 22].

3.2.2 Tolerance to GIT Conditions

The fish intestine consists of a harsh environment with the presence of digestive enzymes, bile salts, and pH variations. Surviving the harsh conditions of the GIT is an important criterion for probiotic selection as it ensures that the bacteria pass through the harsh environment and colonize in the GIT to confer a beneficial effect [23, 24]. Thus, obtaining isolates from the host origin is considered important as these isolates show tolerance to the GIT conditions of the host. Probiotic bacteria must tolerate pH up to 2 and bile salt concentration up to 1% [25]. Tolerance to acid and bile concentrations in broth is used as an initial selection criterion followed by an assessment of the survival capability in simulated GIT fluids.

Acid Tolerance

1. Prepare broth media as per the choice of the organism (MRS for LAB and TSB or LB for other organisms).
2. Adjust the pH of the broth to pH 2 and pH 3 respectively with 0.1 N HCl using a pH meter. Keep broth with neutral pH as growth control.
3. Add the pH-adjusted broth to test tubes and autoclave.
4. Inoculate sterile tubes with overnight grown culture (5%) and incubate at the appropriate temperature.
5. Draw aliquots at regular intervals (0, 1, 2, 3 h) and obtain the plate count by serial dilution and plating.
6. Note down the percentage survival of viable bacteria and calculate by using the following formula:

$$\text{Survival } (\%) = \frac{\text{CFU}_1}{\text{CFU}_0} \times 100$$

where CFU_1 is the bacterial count in the test sample and CFU_0 is the count in the control tube [18, 26].

Bile Tolerance

1. Prepare appropriate broth supplemented with 0.3% and 1% ox-bile, respectively, in test tubes and autoclave.
2. Inoculate the tubes with overnight grown culture (5%) and incubate at the appropriate temperature.
3. Draw aliquots at regular intervals (0, 1, 2, 3 h) and obtain the plate count by serial dilution and plating.
4. Calculate the survival percentage as described in Subheading 3.2.2 [18, 26].

Tolerance to Simulated Gastrointestinal Fluids

1. Prepare simulated gastric juice by dissolving 1000 U/mL of pepsin and 0.01 g/L lysozyme in a sterile buffer containing sodium chloride (2.05 g/L), dihydrogen potassium phosphate (0.60 g/L), calcium chloride (0.11 g/L), and potassium chloride (0.37 g/L). Adjust the pH to 3 with 1 M HCl [27].
2. Prepare simulated intestinal fluid by dissolving 3 g/L of bile salts and 1000 U/mL trypsin in a buffer containing 50.81 g/L disodium hydrogen phosphate, 8.5 g/L of sodium chloride, and 1.27 g/L of dihydrogen potassium phosphate.
3. Take overnight test cultures and harvest the cells by centrifuging at 8000 rpm for 10 min. Wash the cells twice in PBS and resuspend in simulated gastric juice (5%). Incubate at the appropriate temperature.
4. Obtain plate counts at regular intervals as explained in Subheading 3.2.2.
5. Harvest cells from the simulated gastric juice and resuspend in simulated intestinal juice. Obtain plate counts at regular intervals same as in Subheading 3.2.2.
6. Calculate survival percentages as previously described in Subheading 3.2.2.

3.2.3 Bile Salt Hydrolase (BSH) Assay

Bile salt hydrolase is a plate precipitation assay examined by qualitative analysis.

1. Pour appropriate sterile agar media (MRS/TSA/LB) supplemented with 0.5% of taurodeoxycholic acid (TDCA) and 0.037% calcium chloride ($CaCl_2$) into sterile Petri plates and allow to solidify.
2. Streak overnight grown test cultures on the surface of the agar and incubate at the appropriate temperature for 24–72 h.
3. Observation: White precipitation zones around the colonies indicate BSH activity [28].

3.2.4 Adhesion Assays

Adhesion of potential probiotic bacteria to the GIT of the host is a prerequisite for probiotic selection [29]. Several in vitro adhesion assays are used to assess the adhesion ability of potential isolates. Simple assays such as hydrophobicity, auto-aggregation, and co-aggregation provide an initial idea of the isolate to adhere to cells of the same species and other species or competitive adhesion with the pathogen. These assays are often conducted using broth media or buffer solvents. On the other hand, substrates such as intestinal mucus from fish, epithelial cells, and cell lines are also used to demonstrate the adhesion capacity of the isolates [30–32].

Cell Surface Hydrophobicity Assay

This in vitro analysis method is carried out to measure adhesion using microbial adhesion to hydrocarbon (MATH) method [25, 33].

1. Take overnight grown test cultures and centrifuge at 8000 rpm for 10 min at 4 °C to obtain a cell pellet.
2. Wash the cell pellet twice with PUM (phosphate urea magnesium) buffer and resuspend in PUM buffer to obtain a uniform cell suspension.
3. Adjust the initial optical density (OD) at 600 nm to 0.7.
4. Take cell suspension and hydrocarbon (n-hexadecane, xylene, or toluene) in the ratio of 3:1.
5. Vortex the sample well and incubate at 37 °C for 1 h for phase separation.
6. Carefully take the aqueous phase and read the final OD at 600 nm.
7. Calculate the percentage of cell surface hydrophobicity using the following formula:

$$\text{Cell surface hydrophobicity } (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 indicates initial optical density and A_1 indicates final optical density.

Auto-aggregation

1. Harvest overnight grown cultures by centrifugation, wash twice in PBS, and resuspend in PBS to get a uniform cell suspension adjusted to an OD 600 nm of 0.6.
2. Briefly vortex the cell suspension and incubate at appropriate temperature for 24 h.
3. Take readings at regular intervals (0, 2, 4, 6, and 24 h) [22, 26].
4. Calculate the auto-aggregation percentage using the following formula:

$$\text{Auto-aggregation } (\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

where A_0 = initial absorbance and A_1 = final absorbance.

Co-aggregation

1. Cultivate test cultures in appropriate medium and pathogen cultures in BHI broth.
2. Harvest overnight grown cultures as mentioned in auto-aggregation procedure.
3. Mix equal volumes of test culture and pathogen culture in a test tube and incubate at room temperature for 4 h.

4. Keep tubes containing cell suspension of each culture separately as control.
5. Read the absorbance of mixed cultures and monocultures at 600 nm.
6. Calculate co-aggregation percentage using the following formula:

$$\text{Co-aggregation\%} = \left[\frac{(A_t + A_p) - 2A_{mix}}{(A_t + A_p)}\right] \times 100$$

where A_t and A_p are the absorbance of test culture and pathogen suspension, respectively, while A_{mix} is the absorbance of mixed bacterial suspension after incubation [27].

In Vitro Cell Adhesion Assay

(a) **Preparation of Mucus** [24]

1. Collect harvested fish and disinfect with 0.1% benzalkonium chloride solution.
2. Dissect with a scalpel and remove the hindgut.
3. Rinse the inner surface of the hindgut with PBS (pH 7.2) and scrape with a rubber spatula.
4. Centrifuge the scrapings twice at 6000 rpm for 30 min at 4 °C to remove any particulate and debris material.
5. Filter the preparation through a 0.22 μm syringe filter.
6. Determine the concentration of mucus by Bradford method and adjust the protein concentration to 0.5 or 1 mg/mL. Store at −70 °C until further use.

(b) **Adhesion to Mucus** [24, 33]

1. Take a 96-well microtiter plate and coat with 100 μL of mucus. Incubate the plate overnight for adhesion of the mucus at 4 °C.
2. Take overnight grown test cultures and adjust the cell density to 1×10^8 cells/mL (A_0).
3. Add 100 μL of test culture to the immobilized mucus and incubate at 37 °C or appropriate temperature for 1 h.
4. Wash the wells with PBS to remove non-adherent cells.
5. Fix the adhered cells at 60 °C for 20 min and stain with 100 μL crystal violet (0.1% solution) for 15 min.
6. Wash the wells with PBS to remove excess stain.
7. Treat the cells with 100 μL of 20 mM acetate or citrate buffer (pH 4.3) to release the stain bound to the cells.
8. Measure the absorbance at 600 nm (A_1) in a microtiter plate reader.

9. Keep stained mucus without added bacteria as negative control (A_{control}).
10. Calculate the percentage of adherent bacterial cells using the following formula:

$$\text{Adhesion}\% = A_1 - \frac{A_{\text{control}}}{A_0} \times 100$$

3.2.5 Production of Extracellular Enzymes

1. To test proteinase production, inoculate peptone–gelatin agar plates with test cultures and incubate at appropriate temperature for 48 h. Flood the plates with 15% $HgCl_2$. Clear zone around the colony indicates positive reaction for proteinase.
2. To test for amylase production, inoculate starch (1%) agar plates with test cultures and incubate for 24 h at appropriate temperature and subsequently flood with Lugol's iodine solution. Whitish yellow discoloration on the plate is indicative of amylase activity.
3. To test for lipolytic enzyme production, inoculate test cultures onto tributyrin (1%) agar. Whitish opaque zone around the test culture colony indicates lipase activity.
4. To determine cellulose production, inoculate test cultures onto carboxymethyl cellulose agar plates for 48 h at appropriate temperature. Flood the plates with Gram's iodine solution. Presence of whitish appearance around the colony is indicative of cellulose production [33, 34].

Acknowledgments

The authors wish to express their sincere gratitude to the authorities at JSS Academy of Higher Education & Research, Mysuru, for extending necessary support and facilities. ACA gratefully acknowledges the DBT Wellcome Trust India Alliance for grant of Early Career Fellowship (IA/E/20/1/505689). MSP acknowledges the support of Karnataka Govt. for grant of OBC fellowship (OBC-10/PhD/CR-04/2021-22).

References

1. Amoah K, Huang QC, Tan BP, Zhang S, Chi SY, Yang QH, Liu HY, Dong XH (2019) Dietary supplementation of probiotic *Bacillus coagulans* ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 87:796–808
2. Azad MAK, Islam SS, Sithi IN, Ghosh AK, Banu GR, Bir J, Huq KA (2019) Effect of probiotics on immune competence of giant freshwater prawn *Macrobrachium rosenbergii*. Aquac Res 50(2):644–657
3. El-Saadony MT, Alagawany M, Patra AK, Kar I, Tiwari R, Dawood MA, Dhama K, Abdel-Latif HM (2021) The functionality of probiotics in aquaculture: an overview. Fish Shellfish Immunol 117:36–52
4. Balcázar JL, De Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL

(2006) The role of probiotics in aquaculture. Vet Microbiol 114(3–4):173–186

5. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64(4):655–671
6. Kumari VBC, Huligere SS, Ramu R, Naik Bajpe S, Sreenivasa MY, Silina E, Stupin V, Achar RR (2022) Evaluation of Probiotic and Antidiabetic Attributes of Lactobacillus Strains Isolated From Fermented Beetroot. Front Microbiol 13:911243
7. Chemlal-Kherraz D, Sahnouni F, Matallah-Boutiba A, Boutiba Z (2012) The probiotic potential of lactobacilli isolated from Nile tilapia (*Oreochromis niloticus*)'s intestine. Afr J Biotechnol 11(68):13220–13227
8. Balcázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Muzquiz JL, Girones O (2008) Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture 278(1–4):188–191
9. Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, Del Campo R, Hernández PE, Herranz C, Cintas LM (2013) Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol 13(1):1–22
10. Abdel-Latif HM, Khafaga AF (2020) Natural co-infection of cultured Nile tilapia *Oreochromis niloticus* with *Aeromonas hydrophila* and *Gyrodactylus cichlidarum* experiencing high mortality during summer. Aquac Res 51(5): 1880–1892
11. El-Son MA, Nofal MI, Abdel-Latif HM (2021) Co-infection of *Aeromonas hydrophila* and *Vibrio parahaemolyticus* isolated from diseased farmed striped mullet (*Mugil cephalus*) in Manzala, Egypt – a case report. Aquaculture 530: 735738
12. Cota-Gastélum LA, Luna-González A, Gonzalez-Ocampo HA, del Carmen Flores-Mir M, Fierro-Coronado JA, Escamilla-Montes R, Peraza-Gómez V (2013) Effect of *Pediococcus* sp., *Pediococcus pentosaceus*, inulin and fulvic acid, added to the diet, on growth of *Oreochromis niloticus*. Afr J Microbiol Res 7(48):5489–5495
13. Nayak SK, Mukherjee SC (2011) Screening of gastrointestinal bacteria of Indian major carps for a candidate probiotic species for aquaculture practices. Aquac Res 42(7):1034–1041
14. Smibert RM, Krieg NR (1981) General characterization. In: Gerdhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (Eds.) Manual of methods for general bacteriology, American Society for Microbiology, Washington, DC, pp 409–444
15. Buxton R (2005) Blood agar plates and hemolysis protocols. American Society for Microbiology, Washington, DC, pp 1–9
16. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10(6):2740
17. AlKalbani NS, Turner MS, Ayyash MM (2019) Isolation, identification, and potential probiotic characterization of isolated lactic acid bacteria and *in vitro* investigation of the cytotoxicity, antioxidant, and antidiabetic activities in fermented sausage. Microb Cell Factories 18(1):1–12
18. Patel P, Patel B, Amaresan N, Joshi B, Shah R, Krishnamurthy R (2020) Isolation and characterization of *Lactococcus garvieae* from the fish gut for in vitro fermentation with carbohydrates from agro-industrial waste. Biotechnol Rep 28:e00555
19. Ringø E, Schillinger U, Holzapfel W (2005) Antimicrobial activity of lactic acid bacteria isolated from aquatic animals and the use of lactic acid bacteria in aquaculture. In: Biology of growing animals, vol 2. Elsevier, Amsterdam, pp 418–453
20. Deodware SA, Barache UB, Dhale PC, Gaikwad KD, Shivamallu C, Ubale PA, Shati AA, Alfaifi MY, Elbehairi SEI, Achar RR, Silina E, Stupin V, Frau J, Flores-Holguín N, Gaikwad SH, Kollur SP, Glossman-Mitnik D (2022) In vitro anticancer screening, molecular docking and antimicrobial studies of triazole-based Nickel (II) metal complexes. Molecules 27 (19):6548
21. Ringø E, Løvmo L, Kristiansen M, Bakken Y, Salinas I, Myklebust R, Olsen RE, Mayhew TM (2010) Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. Aquac Res 41(4):451–467
22. Govindaraj K, Samayanpaulraj V, Narayanadoss V, Uthandakalaipandian R (2021) Isolation of lactic acid bacteria from intestine of freshwater fishes and elucidation of probiotic potential for aquaculture application. Probiotics Antimicrob Proteins 13(6): 1598–1610
23. Hlophe SN, Moyo NAG, Ncube I (2014) Postprandial changes in p H and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897), *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). J Appl Ichthyol 30(1):35–41

24. Geraylou Z, Vanhove MP, Souffreau C, Rurangwa E, Buyse J, Ollevier F (2014) In vitro selection and characterization of putative probiotics isolated from the gut of Acipenser baerii (Brandt, 1869). Aquac Res 45(2): 341–352
25. Archer AC, Halami PM (2015) Probiotic attributes of *Lactobacillus fermentum* isolated from human feces and dairy products. Appl Microbiol Biotechnol 99(19):8113–8123
26. Samson JS, Choresca CH, Quiazon KMA (2020) Selection and screening of bacteria from African nightcrawler, *Eudrilus eugeniae* (Kinberg, 1867) as potential probiotics in aquaculture. World J Microbiol Biotechnol 36(1):1–10
27. Kaktcham PM, Temgoua JB, Zambou FN, Diaz-Ruiz G, Wacher C, Pérez-Chabela MDL (2018) In vitro evaluation of the probiotic and safety properties of bacteriocinogenic and non-bacteriocinogenic lactic acid bacteria from the intestines of Nile tilapia and common carp for their use as probiotics in aquaculture. Probiotics Antimicrob Proteins 10(1):98–109
28. Gil-Rodríguez AM, Beresford T (2021) Bile salt hydrolase and lipase inhibitory activity in reconstituted skim milk fermented with lactic acid bacteria. J Funct Foods 77:104342
29. Ringø E, Gatesoupe FJ (1998) Lactic acid bacteria in fish: a review. Aquaculture 160(3–4): 177–203
30. Abdulla AA, Abed TA, Saeed AM (2014) Adhesion, autoaggregation and hydrophobicity of six *Lactobacillus* strains. Br Microbiol Res J 4(4):381–391
31. Lazado CC, Caipang CMA, Brinchmann MF, Kiron V (2011) In vitro adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. Vet Microbiol 148(2–4): 252–259
32. Bellon-Fontaine MN, Rault J, Van Oss CJ (1996) Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. Colloids Surf B: Biointerfaces 7(1–2):47–53
33. Khan MIR, Choudhury TG, Kamilya D, Monsang SJ, Parhi J (2021) Characterization of *Bacillus* spp. isolated from the intestine of Labeo rohita—towards identifying novel probiotics for aquaculture. Aquac Res 52(2): 822–830
34. Banerjee S, Ghosh K (2014) Enumeration of gut-associated extracellular enzyme-producing yeasts in some freshwater fishes. J Appl Ichthyol 30(5):986–993

Chapter 16

Antibacterial Activity of Probiotic Bacteria from Aquaculture

Mahalakshmi S. Patil, Anagha Sudhama Jahgirdar, Ann Catherine Archer, and Raghu Ram Achar

Abstract

With a huge demand for antibacterial agents worldwide due to the development of resistance against existing antibiotics, probiotics have gained an upper hand. This extends even to aquaculture production as well and has been considered as a major constraint. As an alternative to antimicrobial agents, the use of probiotic bacteria in aquaculture has gained a lot of attention. This chapter describes in detail the protocols to be followed for effective demonstration and characterization of antibacterial activity of the probiotics in the study. The protocols to be followed, viz., the sample preparation and processing and demonstration of antibacterial and bacteriocin activity, with emphasis on bacteriocin purification and characterization, have been represented.

Key words Antibacterial, Probiotics, Probiotic bacteria, Fish, Aquaculture, Bacteriocins

1 Introduction

Antimicrobial agent refers to any substance that can inhibit the growth of microorganisms or kill it. The aquaculture industry has seen tremendous growth in recent years due to the rising demand for aquatic food globally. However, the production of fish involves significant challenges including production, prevention of spoilage, and disease control [1, 2]. Due to increased production, there is an increase in bacterial and viral diseases in fish. Antibiotics are used widely as prophylactic and therapeutic agents to control diseases, but they are associated with the emergence of antibiotic resistance in bacterial pathogens and its consequent effects on the spread of antibiotic resistance in the food chain [3, 4]. This has prompted the need for new and safe alternatives to antimicrobial compounds. Among these, probiotic bacteria are said to be beneficial microorganisms capable of inhibiting pathogenic bacteria via various mechanisms such as modulation of the gut microbiota, competitive

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_16,

exclusion, and modulation of the immune system [1]. Probiotics also secrete compounds with antimicrobial potential such as enzymes, bacteriocins, organic acids, and hydrogen peroxide [5, 6].

Lactic acid bacteria (LAB) are one of the common groups of microorganisms that have been found to possess probiotic properties due to their long, safe history in food fermentations and presence as natural flora in the gastrointestinal tract of humans, mammals, and even fishes [1, 7, 8]. LAB as probiotics have been found to decrease aquatic diseases caused by pathogens like *Aeromonas salmonicida* and *Vibrio anguillarum* and improve the overall health of the fishes [5, 9, 10]. Thus, LAB become an important source of probiotic bacteria and antimicrobial substances to effectively prevent and control bacterial diseases in fishes [11, 12]. Several techniques have been used to investigate the antagonistic or antimicrobial activity of probiotic LAB [13].

Some of the precise methods including qualitative assay of antimicrobial action to detection of presence, purification, and characterization of bacteriocins have been outlined in this chapter.

2 Materials

Bacterial Strains and Growth

LAB is grown in DeMan Rogosa Sharpe (MRS) media (composition in Table 1). Pathogenic organisms used as indicator strains for antibacterial activity is grown in tryptic soy (TS) or brain heart infusion (BHI) media (composition in Tables 2 and 3). The indicator strains which cause disease in fish are listed in Table 4. Selected strains from the list can be used for antibacterial studies.

Purification and Characterization

Ammonium sulfate, acetonitrile, trifluoroacetic acid, tricine, sodium dodecyl sulfate, acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, tetramethylethylenediamine, Coomassie brilliant blue R-250, silver nitrate, formaldehyde, ethanol, acetic acid, CM Sepharose/SP Sepharose, CM Sephadex G50/75/100, C18 reverse-phase columns.

3 Methodology

3.1 Sample Collection and Processing

1. Collect freshwater or marine water fish from local sources.
2. Weigh the fishes and aseptically remove the intestines, place in ice, and transport to the laboratory.
3. Homogenize the fish intestines and its contents in 10 mL phosphate-buffered saline.

Table 1
Composition of MRS media

Ingredients	Grams/liter
Peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2
Agar	12
pH	6.5

Table 2
Composition of tryptic soy media

Ingredients	Grams/liter
Pancreatic digest of casein	15
Peptic digest of soybean meal	5
Sodium chloride	5
Agar	15
pH	7.3

Table 3
Composition of brain heart infusion media

Ingredients	Grams/liter
HM infusion powder	12.5
BHI powder	5
Peptone	10
Dextrose	2
Sodium chloride	5
Disodium hydrogen phosphate	2.5
Agar	15
pH	7.4

Table 4
Common bacterial fish pathogens that can be used as indicator strains for antibacterial activity

Pathogen species	Host	Disease caused
Gram-negative species		
Aeromonas hydrophila	Trout, goldfish, salmon, koi, and other fishes	Furunculosis
Aeromonas salmonicida	Trout, goldfish, salmon, koi, and other fishes	Furunculosis
Aeromonas veronii	Catfish, tilapia, striped bass, sturgeon, eel, salmonid, and non-salmonid species	Motile aeromonas septicemia (MAS), hemorrhagic septicemia, epizootic ulcerative syndrome (EUS), red sore disease
Pseudomonas anguilliseptica	Eel, turbot, sea bream, ayu	Pseudomonadiasis, winter disease
Yersinia ruckeri	Salmonids, sturgeon, crustaceans, eel, minnows	Enteric red mouth
Piscirickettsia salmonis	Salmonids	Piscirickettsiosis
Edwardsiella ictaluri	Catfish and tilapia	Enteric septicemia
Edwardsiella tarda	Tilapia, catfish, salmon, carps, yellowtail, flounder, striped brass	Edwardsiellosis
Pasteurella skyensis	Salmonids and turbot	Pasteurellosis
Vibrio anguillarum	Turbot, salmonids, striped bass, sea bass, eel, cod, ayu, red sea bream	Vibriosis
Aliivibrio salmonicida	Cod, Atlantic salmon	Vibriosis
Vibrio vulnificus	Tilapia, eel	Vibriosis
Vibrio ordalii	Salmonids	Vibriosis
Vibrio harveyi	Abalone, shark, sea bream, red drum, cobia, sea bass, flounder	Vibriosis
Photobacterium damselae	Sea bass, sea bream, striped bass, yellowtail, sole	Photobacteriosis
Flavobacterium psychrophilum	Carp, salmonids, eel, perch, ayu	Coldwater disease
Flavobacterium branchiophila	Cold water and warm water salmonid and non-salmonid species	Bacterial gill disease
Flavobacterium columnare		

(continued)

Table 4 (continued)

Pathogen species	Host	Disease caused
Gram-positive species		
Lactococcus garvieae	Yellowtail, rainbow trout, prawns, turbot, and eel	Streptococcosis or lactococcosis
Streptococcus iniae	Yellowtail, sea bass, barramundi, flounder	Streptococcosis
Streptococcus parauberis	Turbot	Streptococcosis
Streptococcus phocae	Atlantic salmon	Streptococcosis
Enterococcus faecalis	Rainbow trout, brown bullhead, catfish	Streptococcosis, exophthalmia hemorrhage
Other common food pathogens		
Listeria monocytogenes	–	–
Escherichia coli	–	–
Staphylococcus aureus	–	–

4. Serially dilute the homogenate in sterile saline solution (0.85%) and pour plate appropriate dilutions on DeMan Rogosa Sharpe (MRS) agar in sterile Petri dishes.
5. Incubate the plates at 25–30 °C (depending on the source of the sample) for 24–48 h.
6. Pick single colonies based on morphology characteristics, grow in MRS broth, and purify by streak plate technique.
7. Purified isolates are identified as LAB by Gram staining and catalase test.
8. The isolates are stored as 40% glycerol stock at −20 °C.

3.2 Agar Overlay Assay

Agar overlay assay is a method of direct antimicrobial activity testing. It is also called the double agar layer method. This can be done by two methods:

3.2.1 Method-I

1. Prepare serial dilutions of fish intestine samples as explained in Subheading 3.1, pour plate with MRS agar, and incubate at 25–30 °C for 24–48 h.
2. Once the colonies of LAB are formed, pour a layer of tryptic soy or BHI soft agar (0.8%) seeded with indicator pathogen

organism (1% inoculum) over the plate and allow to solidify [14].

3. Incubate the plates at 37 °C for growth of the indicator organism.
4. After incubation, a clear zone of inhibition around the colonies of LAB indicates antibacterial activity.

3.2.2 Method-II

1. Prepare fresh cultures of purified LAB isolates by inoculating LAB in MRS broth and incubating overnight.
2. Place 5–10 μL spots of LAB cultures on a layer of MRS agar at appropriate distances.
3. Incubate the plates at 25–30 °C to allow the spots to grow.
4. Overlay the plate with tryptic soy (TS) or BHI soft agar (0.8%) seeded with indicator pathogen organism and allow to solidify.
5. Incubate the plates at 37 °C for growth of the indicator organism.
6. After incubation, a clear zone of inhibition around the colonies of LAB (Fig. 1) indicates antibacterial activity [15].

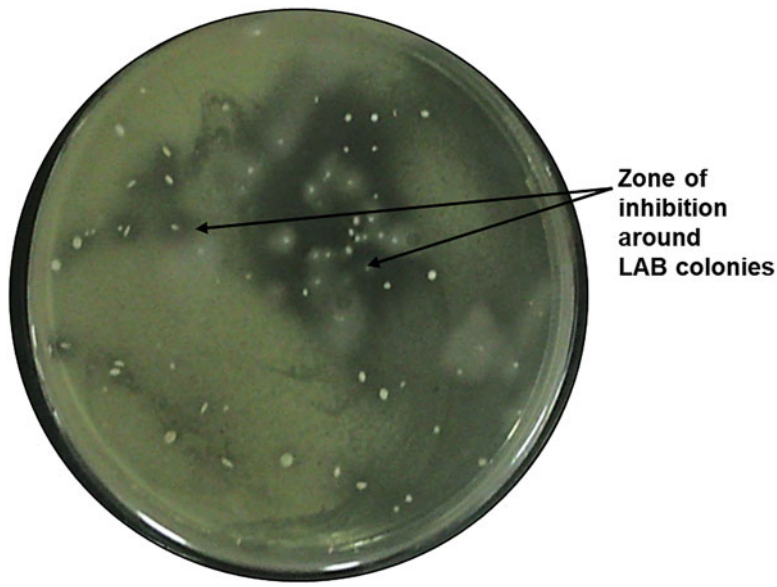

Fig. 1 Representative plate showing agar overlay assay. Serial dilution of fish intestine sample is first plated in MRS agar. Colonies developed after incubation are overlayed with soft agar seeded with indicator pathogenic organism. Development of inhibition zone around the LAB colonies is indicative of antibacterial activity. (*This unpublished data belongs to Dr. Ann Catherine Archer*)

3.3 Cell-Free Supernatant (CFS) Preparation

1. Freshly culture LAB isolates in MRS broth for 18–24 h.
2. Cell-free supernatant is obtained by centrifuging the cultures at 8000 rpm for 10 min.
3. Filter the CFS through 0.22 μm filter and store at −20 °C until further use [16].

3.4 Antibacterial Activity by Agar Well Diffusion Assay

The antibacterial activity of LAB isolates is tested by agar well diffusion assay against common fish pathogens (Table 1) used as indicator organisms [17, 18].

1. Prepare 0.8% soft agar (w/v) of tryptic soy media or brain heart infusion media.
2. Seed 1% of each indicator organism (1×10^5 CFU/mL) into the soft agar separately, mix well, and pour into sterile Petri dishes.
3. Allow the media to solidify.
4. Bore 6–8 mm wells onto the seeded agar using a cork borer or a sterile tip. Label the wells on the bottom of the plate with the respective LAB isolates.
5. Add 50–100 μL of LAB CFS in the wells, and allow the CFS to diffuse through the wells for 1–2 h at room temperature.
6. Incubate the plates at 37 °C or appropriate temperature of the indicator strain for 24 h.
7. Observe the formation of zone of inhibition around the wells which indicates antibacterial activity of the CFS (Fig. 2).

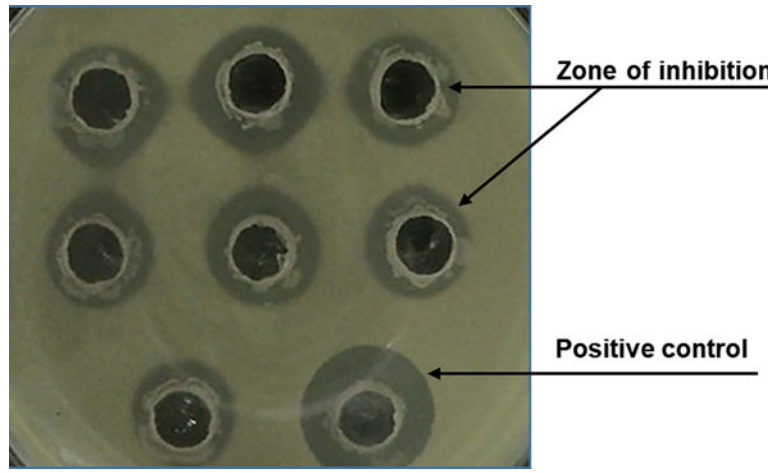

Fig. 2 Representative plate showing agar well diffusion. CFS of LAB isolates are added to wells bored into soft agar seeded with indicator pathogen. CFS is allowed to diffuse through the agar resulting in the formation of zone of inhibition indicative of antimicrobial activity. (*This unpublished data belongs to Dr. Ann Catherine Archer*)

8. Measure the zone diameter using a scale and record the results in mm.
9. The antibacterial activity may be due to production of organic acids or antimicrobial compounds such as bacteriocins.

3.4.1 Determination of Bacteriocin Activity

1. To confirm if the inhibition activity is due to production of bacteriocins, the CFS is neutralized with 1 M NaOH [19].
2. The neutralized CFS is tested for antibacterial activity by well diffusion assay as described in Subheading 3.4.

3.5 Growth Dynamics and Bacteriocin Production

1. Prepare 100 mL MRS broth in a conical flask and autoclave at 121 °C for 20 min.
2. Inoculate LAB isolate at 1% into the broth and incubate at 30–37 °C for 48 h.
3. Take aliquot of sample at regular intervals (0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 h).
4. Determine the cell density at 600 nm, pH of the culture, and antibacterial activity by well diffusion assay.
5. The activity is expressed as arbitrary units per milliliter of culture medium (AU/mL).
6. One AU/mL is defined as the reciprocal of the highest dilution of bacteriocin showing a clear zone of inhibition against the indicator strain.

AU per milliliter was calculated with the following formula [20, 21]:

$$\frac{\text{AU}}{\text{mL}} = \frac{\text{Reciprocal of highest dilution}}{\text{Amount of Bacteriocin used}} \times 1000$$

Also mentioned as

$$\frac{\text{AU}}{\text{mL}} = \left(\frac{1000}{V \times D}\right)$$

where V is the volume of the sample and D is the highest dilution factor.

or

some researchers rely on a recently modified equation [22]:

$$\frac{\text{AU}}{\text{mL}} = a^b \times 100$$

where $a = 2$ (factor dilution) and $b =$ value of the highest dilution showing at least 2 mm inhibition zone.

3.6 Broth Microdilution Assay

Broth microdilution assay can be done using crude CFS or purified bacteriocin extract to determine the minimum inhibitory concentration (MIC) of the inhibitory compound. The procedure involves serial dilutions of the test sample (crude CFS/purified bacteriocin) and incubating with the indicator organism. MIC is the lowest concentration of antimicrobial agent that can visibly inhibit the growth of the organism [23].

1. Add 200 μL volume of test sample containing 100 μL of neutralized CFS or purified bacteriocin of LAB isolates and 100 μL of indicator organism (10^5–10^6 CFU/mL) in TS/BHI broth into the first well of a microtiter plate.
2. Transfer 100 μL of sample from the first well into the second well containing 100 μL of indicator organism in TS/BHI broth.
3. Repeat the dilution for subsequent wells as described in **step 3**.
4. Incubate the plate at 37 °C for 24 h.
5. MIC is determined by measuring the optical density of the test wells at 600 nm.
6. Plate aliquot of each dilution to determine the viable count of indicator organism.

3.7 Time-Kill Assay

Time-kill assay is a method to determine the bactericidal effect of CFS. It is a time-dependent method to study the bactericidal activity of LAB against the growth dynamics of the indicator organism [24, 25].

1. Prepare tryptic soy or BHI broth (10 mL) in test tubes and autoclave the tubes at 121 °C for 20 min. Allow the tubes to cool.
2. Inoculate the tubes with indicator organism (10^5 CFU/mL) and add 100 μL of crude CFS to the tubes.
3. Incubate the tubes at 37 °C for 24 h.
4. Draw aliquots at regular intervals (0, 2, 4, 6, 8, 10, 12, 24 h) and determine the CFU/mL by plate count method.
5. Tubes inoculated with only indicator organism are kept as growth control.
6. Bactericidal effect is determined by plotting the graph of viable count against time and comparing the growth of indicator organisms in the control and test samples.

3.8 Time-Kill Co-culture Assay

This is a method to determine the antibacterial activity of LAB isolates by co-culturing with the indicator organism [26].

1. Culture LAB isolates and indicator organisms individually in their respective broth mediums.

2. The cultures are centrifuged at 8000 rpm for 10 min to obtain cell pellet.
3. Inoculate indicator organism (10^5–10^6 CFU/mL) with 10^6–10^8 CFU/mL of LAB isolates in 10 mL MRS-TS/BHI broth (1:1 ratio).
4. Incubate the co-culture tubes at 37 °C. Aliquot samples at regular intervals (0, 2, 4, 6, 8, 10, 12, 24 h) and determine the viable count by plate count method (MRS agar for LAB and TS/BHI agar for indicator strains). Also measure the pH changes.
5. Tubes inoculated with monocultures of LAB or indicator organism are kept as control.
6. Bactericidal effect is determined by plotting graph of the viable count and comparing with growth control.

3.9 Scanning Electron Microscopy

1. Incubate crude CFS and indicator organism as described in the time-kill assay. Take aliquots at 30, 90, and 120 min. Centrifuge the samples at 8000 rpm and wash the cell pellet with PBS [27].
2. The cell pellet is fixed by adding 2.5% glutaraldehyde and incubated at 4 °C overnight [28].
3. Next day, wash the cells with PBS and dehydrate by incubating the cells in increasing concentrations of ethanol (5, 10, 20, 30, 50, 70, 90, 100%).
4. Aliquot of the final cell suspension after dehydration in 100% ethanol is placed on a coverslip and allowed to dry.
5. The coverslip is subjected to gold sputter coating. The coated samples are visualized under scanning electron microscope. Modified newer methods have been adopted over time with variety of fixative based on the desired magnification and resolution [29].
6. Morphological changes in cell shape and structure are observed in the samples treated with CFS [30].

3.10 Purification of Bacteriocin

1. The cell-free supernatants are centrifuged at 8000 to 10,000×*g* for 10–20 min at 4 °C to remove debris. Then, addition of ammonium sulfate from 10% to 80% saturation is done to precipitate the bulky proteins with stirring at 4 °C. The obtained saturation is centrifuged at 8000 to 10,000×*g* for 10–20 min at 4 °C to obtain fractions as pellets containing bacteriocin [31].
2. The bacteriocin pellets are then resuspended in appropriate buffers or diluted using distilled water. These initial crude extracts are subjected to agar well diffusion method as mentioned in Subheading 3.4.

3. The crude extracts are further purified using gel filtration or ion exchange as per the standard protocols [32].
4. Briefly, cation exchange chromatography can be performed with UV detection at 220 nm. One liter of the suspension obtained from precipitation is into a strong cation exchange resin column and subjected to elution at a flow rate of 1.5–2 mL/min and washed with 3× column volume of appropriate buffer at the flow rate of 1 mL/min. The obtainment of bacteriocin fractions must be standardized based on the different concentration of NaCl from 0.2 to 1 M [33]. The active fraction is lyophilized and stored at −20 °C for further purification and analyses.
5. Furthermore, the active fractions of bacteriocins are subjected to gel filtration by loading it onto a Sephadex column of required specification. The choice is ideally based on the established recommendations [34]. Ideally, the column is equilibrated with the chosen appropriate buffer used for suspension. The column is eluted with the same buffer and the fractions are collected at 1.0–1.5 mL/min and the absorbance was 220 and 280 nm. The collected fractions are again evaluated for antibacterial activity as described by the abovementioned procedures, and the active fractions are dialyzed in distilled water for further purification.
6. The active fraction purified from gel filtration chromatography process is processed further onto a C18 reverse-phase columns. Reverse phase high-performance liquid chromatography (RP-HPLC) system by a liner gradient elution would provide confirmation of purity. The elution is with the standard eluent, i.e., 95% water–acetonitrile containing 0.1% trifluoroacetic acid (TFA). The flow rate is usually fixed to 0.5 mL/min and may be extended up to 1 mL/min [35].
7. Note: The concentration of protein in every process of purification. The collected fractions after every purification step are pooled based on the peaks obtained using UV–Vis spectroscopy at 280 and 220 nm and evaluated for antibacterial activity as described by the abovementioned procedures.

3.11 Characterization of Bacteriocin

3.11.1 Determination of Molecular Weight

1. Initially, tricine SDS–PAGE analysis of fractions from CFS to the purified fraction after each purification is subjected to 16–18% resolving gel (subject to the equipment and resolution achieved) and 4% stacking gel.
2. The gel is subjected to staining with Coomassie brilliant blue R-250 or silver staining for molecular weight using standard ladder (between 2 and 60 kD). One more unstained part of the gel should be subjected to 20% (v/v) isopropanol and 10% (v/v) acetic acid for 2–3 h and rinsed in sodium phosphate buffer (pH 6.0) for 1 h [36].

3. The resultant bands are then overlaid with soft agar medium inoculated with the indicator strain for 24–48 h for antibacterial activity as per the abovementioned protocol in Subheading 3.4.
4. Mass spectrometry analyses are carried out using LC/MS system. Briefly, by following the abovementioned steps of liquid chromatography which is linked to the mass spectrometer is programmed to run in positive electron spray ionization (ESI) mode with mass/charge (m/z) ratio in the range of m/z (based on the relative molecular weight obtained in PAGE analysis) and a quadrupole time of flight (Q-TOF) mass analyzer is ideally used [37].

3.11.2 Sensitivity of Probiotic-Generated Antibacterials to Microenvironments

(a) *Heat:* The purified fractions are subjected to a range of incubation temperatures from 25 to 121 °C.

(b) *pH:* The probiotics and purified fractions are subjected to a range of buffers—usually between 2 and 12 for a minimum of 1 h and may be extended up to 2–3 h with occasional stirring at 37 °C.

(c) *Physiological Enzymes:* The probiotics or their extracts or the generated metabolites will have to be subjected to inactivation by the treatment of physiological enzymes like catalase, trypsin, pepsin, and proteinase K for 1–5 h at 37 °C with constant stirring.

Note: The treated fractions mentioned above are subjected to antibacterial activity as mentioned in Subheading 3.4.

Acknowledgments

The authors wish to express their sincere gratitude to the authorities at JSS Academy of Higher Education & Research, Mysuru, for extending necessary support and facilities. ACA gratefully acknowledges the DBT Wellcome Trust India Alliance for grant of Early Career Fellowship (IA/E/20/1/505689). MSP acknowledges the support of Karnataka Govt. for OBC fellowship (OBC-10/PhD/CR-04/2021-22).

References

1. Archer AC, Halami PM (2015) Probiotic attributes of Lactobacillus fermentum isolated from human feces and dairy products. Appl Microbiol Biotechnol 99:8113–8123
2. Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, del Campo R, Hernández PE et al (2013) Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol 13:15
3. Alonso S, Carmen Castro M, Berdasco M, de la Banda IG, Moreno-Ventas X, de Rojas AH (2019) Isolation and partial characterization of lactic acid bacteria from the gut microbiota of marine fishes for potential application as probiotics in aquaculture. Probiotics Antimicrob Proteins 11:569–579

4. Agarwal P, Kayala P, Chandrasekaran N, Mukherjee A, Shah S, Thomas J (2021) Antioxidant and antibacterial activity of Gelidium pusillum (Stackhouse) against Aeromonas caviae and its applications in aquaculture. Aquac Int 29:845–858
5. Yi Y, Zhang Z, Zhao F, Liu H, Yu L, Zha J et al (2018) Probiotic potential of Bacillus velezensis JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on Carassius auratus. Fish Shellfish Immunol 78:322–330
6. Garcés ME, Olivera NL, Fernández M, Rossi CR, Sequeiros C (2020) Antimicrobial activity of bacteriocin-producing Carnobacterium spp. isolated from healthy Patagonian trout and their potential for use in aquaculture. Aquac Res 51:4602–4612
7. Wang G, Jin X (2019) Lactic acid bacteria in animal breeding and aquaculture. In: Chen W (ed) Lact Acid Bact Bioeng Ind Appl. Singapore: Springer, pp. 257–283.
8. Kumari VBC, Huligere SS, Ramu R, Naik Bajpe S, Sreenivasa MY, Silina E, Stupin V, Achar RR (2022) Evaluation of Probiotic and Antidiabetic Attributes of Lactobacillus Strains Isolated From Fermented Beetroot. Front Microbiol 13:911243
9. Wang Y, Wang J, Bai D, Wei Y, Sun J, Luo Y et al (1862) Synergistic inhibition mechanism of pediocin PA-1 and L-lactic acid against Aeromonas hydrophila. Biochim Biophys Acta BBA Biomembr 2020:183346
10. Zoumpourtikoudi V, Pyrgelis N, Chatzigrigoriou M, Tasakis RN, Touraki M (2018) Interactions among yeast and probiotic bacteria enhance probiotic properties and metabolism offering augmented protection to Artemia franciscana against Vibrio anguillarum. Microb Pathog 125:497–506
11. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671
12. Hoseinifar SH, Sun YZ, Wang A, Zhou Z (2018) Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. Front Microbiol 9:2429
13. Ringø E, Schillinger U, Holzapfel W (2005) Chapter 18. Antimicrobial activity of lactic acid bacteria isolated from aquatic animals and the use of lactic acid bacteria in aquaculture. In: Holzapfel WH, Naughton PJ, Pierzynowski SG, Zabielski R, Salek E (eds). Biol Grow Anim 2:418–453
14. Drolia R, Amalaradjou MAR, Ryan V, Tenguria S, Liu D, Bai X et al (2020) Receptor-targeted engineered probiotics mitigate lethal Listeria infection. Nat Commun 11:6344
15. Arakawa K (1887) Basic antibacterial assay to screen for bacteriocinogenic lactic acid bacteria and to elementarily characterize their bacteriocins. Methods Mol Biol (Clifton NJ) 2019:15–22
16. Koohestani M, Moradi M, Tajik H, Badali A (2018) Effects of cell-free supernatant of Lactobacillus acidophilus LA5 and Lactobacillus casei 431 against planktonic form and biofilm of Staphylococcus aureus. Vet Res Forum 9: 301–306
17. Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 6:71–79
18. Jini R, Swapna HC, Rai AK, Vrinda R, Halami PM, Sachindra NM, Bhaskar N (2011) Isolation and characterization of potential lactic acid bacteria (LAB) from freshwater fish processing wastes for application in fermentative utilisation of fish processing waste. Braz J Microbiol 42:1516–1525
19. Saltaji S, Rué O, Sopena V, Sablé S, Tambadou F, Didelot S et al (2020) Lactococcus lactis diversity revealed by targeted amplicon sequencing of purR gene, metabolic comparisons and antimicrobial properties in an undefined mixed starter culture used for soft-cheese manufacture. Foods 9:622
20. Ansari A, Zohra RR, Tarar OM, Qader SAU, Aman A (2018) Screening, purification and characterization of thermostable, protease resistant Bacteriocin active against methicillin resistant Staphylococcus aureus (MRSA). BMC Microbiol 18:192
21. Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991) Detection and activity of a bacteriocin produced by Leuconostoc mesenteroides. Appl Environ Microbiol 57: 3450–3455
22. Gutiérrez-Cortés C, Suarez H, Buitrago G, Nero LA, Todorov SD (2018) Characterization of bacteriocins produced by strains of Pediococcus pentosaceus isolated from Minas cheese. Ann Microbiol BioMed Central 68: 383–398
23. Perales-Adán J, Rubiño S, Martínez-Bueno M, Valdivia E, Montalbán-López M, Cebrián R, Maqueda M (2018) LAB bacteriocins controlling the food isolated (Drug-Resistant) Staphylococci. Front Microbiol 9:1143
24. Prabhurajeshwar C, Chandrakanth K (2019) Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic lactobacilli strains isolated from commercial yoghurt. Clin Nutr Exp 23: 97–115

25. Appiah T, Boakye YD, Agyare C (2017) Antimicrobial activities and time-kill kinetics of extracts of selected Ghanaian mushrooms. Evid Based Complement Altern Med 2017: 4534350
26. Chen CC, Lai CC, Huang HL, Huang WY, Toh HS, Weng TC, Chuang YC, Lu YC, Tang HJ (2019) Antimicrobial activity of lactobacillus species against carbapenem-resistant enterobacteriaceae. Front Microbiol 10:789
27. Robben C, Witte AK, Schoder D, Stessl B, Rossmanith P, Mester P (2019) A fast and easy ATP-based approach enables MIC testing for non-resuscitating VBNC pathogens. Front Microbiol 10:1365
28. Graham L, Orenstein JM (2007) Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat Protoc 2:2439–2450
29. Dassanayake RP, Falkenberg SM, Stasko JA, Shircliff AL, Lippolis JD, Briggs RE (2020) Identification of a reliable fixative solution to preserve the complex architecture of bacterial biofilms for scanning electron microscopy evaluation. PLoS One 15:e0233973
30. Gomes LC, Mergulhão FJ (2017) SEM analysis of surface impact on biofilm antibiotic treatment. Scanning 2017:e2960194
31. Lei S, Zhao R, Sun J, Ran J, Ruan X, Zhu Y (2020) Partial purification and characterization of a broad-spectrum bacteriocin produced by a lactobacillus plantarum zrx03 isolated from infant's feces. Food Sci Nutr 8:2214–2222
32. Doonan S, Cutler P (2004) General strategies. In: Cutler P (ed) Protein Purif Protoc [Internet]. Totowa: Humana Press. pp. 1–13
33. Selkirk C (2004) Ion-exchange chromatography. In: Cutler P (ed) Protein Purif Protoc [Internet]. Totowa: Humana Press. pp. 125–131
34. Ó'Fágáin C, Cummins PM, O'Connor BF (2010) Gel-filtration chromatography. Protein Chromatogr 681:25–33
35. Lardeux H, Duivelshof BL, Colas O, Beck A, McCalley DV, Guillarme D et al (2021) Alternative mobile phase additives for the characterization of protein biopharmaceuticals in liquid chromatography – mass spectrometry. Anal Chim Acta 1156:338347
36. Zhang J, Yang Y, Yang H, Bu Y, Yi H, Zhang L et al (2018) Purification and partial characterization of bacteriocin Lac-B23, a novel bacteriocin production by lactobacillus plantarum J23, isolated from Chinese traditional fermented milk. Front Microbiol 9:2165
37. Sahoo TK, Jena PK, Patel AK, Seshadri S (2015) Purification and molecular characterization of the novel highly potent bacteriocin TSU4 produced by Lactobacillus animalis TSU4. Appl Biochem Biotechnol 177:90–104

Part III

Methods for Treating Infections in Fishes and Shrimps

Chapter 17

Preparation of Marine Algal (Seaweed) Extracts and Quantification of Phytocompounds

S. Thanigaivel, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

Protocol for the seaweed extraction is discussed here which focuses on the different types of algal collections, processing, and preparation of seaweed extract which is obtained through the standard protocol. Aquaculture health management necessitates a steady increase in output by reducing economic losses and using a variety of natural plant-based bioproducts for treating pathogenic bacteria. Hence, such natural product-based extract can be recommended to overcome the side effects of antibiotic use in fisheries sector. According to the current method, bioactive compounds present in two seaweeds are identified through the use of different types of differential extraction methods (sequential and maceration), as well as traditional and solvent-based extraction methods. The extracts with bioactive compounds having antioxidants and antibacterial activities will be effective against fish pathogens.

Key words Seaweeds, Phytochemicals, Extract preparation, Maceration, Sequential, Fish pathogen

1 Introduction

Efficacious disease management with the aid of vaccines, as well as the efficient formulation of extracts and emulsion preparations, is essential for the successful production of aquatic animals and their products in the aquaculture industry [1]. This can be achieved through efficient disease management with the aid of vaccines, as well as the efficient formulating and formulation of extracts and emulsion preparation in the aquaculture industry. It is currently being used to treat and control infections in aquaculture, rather than commercial antibiotics, which promote resistance to pathogenic bacteria. Plant-based extracts are being used to treat and control infections in aquaculture [2]. Furthermore, the consumption of antibiotic-treated fish by humans can result in health problems. Furthermore, it impairs the fish's ability to defend itself against opportunistic pathogenic microorganisms by weakening

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_17,

its immune system. Because of their antibacterial activity against both gram-negative and gram-positive pathogens, seaweeds and their extracts have gained growing attention for use in medicine, biotechnology, and food preservation [3]. Algal extracts contain polysaccharides, proteins, polyunsaturated fatty acids (PUFAs), pigments, polyphenols, flavonoids, cinnamic acid, isoflavones, benzoic acid, lignans, quercetin, minerals, and plant development hormones, among other substances [4]. As a starting material for the production of algal extracts, the seaweed biomass could be utilized [5, 6]. Extraction is the most important and first step in the process of isolating different types of components. It is possible that the extraction efficiency of seaweed will be reduced due to the presence of a complex cell wall, and this will be influenced by the solvent composition, temperature, duration of treatment, and pH. Plant material has been isolated and studied using a variety of techniques, including microwave-assisted extraction, supercritical fluid extraction with carbon dioxide as a solvent, Soxhlet extraction, enzyme-assisted extraction, and ultrasound-assisted extraction [6, 7]. There are a variety of solvents that can be used in this process, including ethanol, acetone, methanol–toluene, methanol–toluene–methanol, petroleum ether, ethyl acetate, dichloromethane, and butanol, to name a few. The employment of these processes necessitates the use of expensive and potentially toxic solvents [8, 9]. To overcome the limits of typical extraction methods and produce algal extract, we used boiling and soaking extraction operations with distilled water as our method of choice [10, 11].

2 Materials

2.1 Reagents and Raw Materials Required

- Marine algae—brown and green seaweeds.
- Distilled water.
- Plastic containers and jars.
- Plastic trays.
- Petri dish.
- Mixer grinder.
- Filtering funnel.
- Conical flask.
- Ascorbic acid.
- Folin–Ciocalteu reagent.
- Gallic acid.
- Quercetin.
- Ethylene disodium tetra acetic acid.
- Ethanol, dichloromethane, petroleum ether, and chloroform.

2.2 Collection of Seaweeds

- Collect the seaweed and process it.
- Wash the seaweeds to remove the debris and salt impurities.
- Dry the seaweeds at a moisture level up to 15%.
- Ground the plants to a particle size of 0.3 mm [9, 12].

3 Methods

3.1 Processing of Seaweed Extract

- Remove epiphytes and other microbial contaminants.
- Shade dry the seaweeds before being used for extraction [9, 13].
- Pulverize dried seaweed leaves.
- Mix them with 100 mL water and agitate the mixture for 24–48 h.
- Filter the extract through a cheesecloth.
- Centrifuging the filtrate for 10 min at 7000 rpm.
- Collect the supernatant and preserve [14].

3.2 Extract Preparation (Conventional Method)

- Maceration methods used for extraction.
- Add 5 g of powdered seaweeds to 100 mL of water and keep overnight in shaker.
- Filter the extract using Whatman filter paper.
- Extracts filtered out and stored for the assays [15].
- Evaporate the extracts under low pressure in a rotary evaporator.
- Dry the samples scraped out.
- Add scraped samples and mix with appropriate Milli-Q water.
- Measure the dry weight of the sample and calculate the dried yields [15].

3.3 Phytochemical Detection in Seaweed Extracts [9]

The following standard protocols were used to conduct phytochemical analysis of plant extract. The following phytochemical screening assays were performed on the plant extracts.

3.3.1 Alkaloids

- Add 1 mL of Dragendorff"s reagents.
- Add 2 mL of seaweed extract.
- Observe the orange–red precipitation.
- Confirm the alkaloid formation.
- The presence of alkaloids is indicated by the presence of green or white precipitate.

3.3.2 Flavonoids

- Add 1 mL of seaweed extract.
- Add 0.1 mL of chloroform.
- Add equal volume of sulfuric acid to the same tube.
- Observed the upper layer for red color formation.
- Confirm the presence of flavonoids.

3.3.3 Carbohydrates

- Add 2 mL plant extract.
- Add 1 mL Molisch's reagent.
- Then add few drops of concentrated sulfuric acid.
- Carbohydrates are indicated by a purple or reddish tint.

3.3.4 Test for Quinine

- Take 1 mL of seaweed extracts.
- Add 1 mL concentrated sulfuric acid.
- Observe the formation of a red tint.
- Confirm the quinone.

3.3.5 Test for Glycosides

- Add 2 mL plant extract.
- Add 3 mL chloroform, followed by 10% ammonia solution.
- Observe the formation of a pink tint.
- Confirm the glycosides.

3.3.6 Test for Triterpenes

- Take 1.5 mL of seaweed extract.
- Add 1 mL Liebermann–Burchard reagent.
- Observe the formation of a blue–green color in the tube.
- The presence of triterpenoids confirmed.

3.3.7 Phenolics

- Take 1 mL plant extract.
- Add 2 mL of distilled water.
- Add few drops of 10% ferric chloride.
- Observe blue or green tint.
- The presence of phenols is confirmed.

3.3.8 Proteins

- Take 1 mL of extracted seaweed.
- Add few drops of ninhydrin.
- Observe the blue color formation.
- Confirm the presence of protein in the extract.

3.3.9 Phytosteroids and Steroids

- Take 1 mL of plant extract in a test tube.
- Add equal volume of chloroform.
- Add few drops of sulfuric acid.
- Observe the brown ring.
- Confirm the phytosteroid.

3.3.10 Phlobatannins

- Take 1 mL of plant extract.
- Add 2% hydrochloride.
- Observe the red-colored precipitate.
- Confirm the presence of phlobatannin.

3.3.11 Anthraquinones

- Prepare 1 mL of plant extract.
- Add few drops of 10% ammonia solution.
- Observe the pink-colored precipitate.
- Confirm the presence of anthraquinones.

References

1. Miccoli A, Manni M, Picchietti S, Scapigliati G (2021) State-of-the-art vaccine research for aquaculture use: the case of three economically relevant fish species. Vaccines 9:140. https://doi.org/10.3390/VACCINES9020140
2. Dawood MAO, Koshio S, Esteban MÁ (2018) Beneficial roles of feed additives as immunostimulants in aquaculture: a review. Rev Aquac 10:950–974. https://doi.org/10.1111/RAQ.12209
3. Masoomi Dezfooli S, Gutierrez-Maddox N, Alfaro A, Seyfoddin A (2019) Encapsulation for delivering bioactives in aquaculture. Rev Aquac 11:631–660. https://doi.org/10.1111/RAQ.12250
4. Yu YY, Chen WD, Liu YJ, Niu J, Chen M, Tian LX (2016) Effect of different dietary levels of Gracilaria lemaneiformis dry power on growth performance, hematological parameters and intestinal structure of juvenile Pacific white shrimp (Litopenaeus vannamei). Aquaculture 450:356–362. https://doi.org/10.1016/j.aquaculture.2015.07.037
5. Abu-Elala NM, Samir A, Wasfy M, Elsayed M (2019) Efficacy of injectable and immersion polyvalent vaccine against streptococcal infections in Broodstock and offspring of Nile tilapia (Oreochromis niloticus). Fish Shellfish Immunol 88:293–300. https://doi.org/10.1016/J.FSI.2019.02.042
6. Thanigaivel S, Chandrasekaran N, Mukherjee A, Thomas J (2016) Seaweeds as an alternative therapeutic source for aquatic disease management. Aquaculture 464:529–536. https://doi.org/10.1016/J.AQUACULTURE.2016.08.001
7. Thanigaivel S, Thomas J, Vickram AS, Anbarasu K, Karunakaran R, Palanivelu J et al (2021) Efficacy of encapsulated biogenic silver nanoparticles and its disease resistance against Vibrio harveyi through oral administration in Macrobrachium rosenbergii. Saudi J Biol Sci 28:7281. https://doi.org/10.1016/J.SJBS.2021.08.037
8. Thanigaivel S, Thomas J, Vickram AS, Gulothungan G, Nanmaran R, Rani DJ (2021) Antioxidant and antibacterial efficacy of Chaetomorpha linum and its toxicological evaluation for the prophylactic treatment against Pseudomonas aeruginosa infection in Labeo rohita. https://doi.org/10.1080/10454438.2021.1961966
9. Thanigaivel S, Vijayakumar S, Gopinath S, Mukherjee A, Chandrasekaran N, Thomas J (2015) In vivo and in vitro antimicrobial activity of Azadirachta indica (Lin) against Citrobacter freundii isolated from naturally infected tilapia (Oreochromis mossambicus). Aquaculture 437:252–255. https://doi.org/10.1016/J.AQUACULTURE.2014.12.008

10. Schleder DD, Peruch LGB, Poli MA, Ferreira TH, Silva CP, Andreatta ER et al (2018) Effect of brown seaweeds on Pacific white shrimp growth performance, gut morphology, digestive enzymes activity and resistance to white spot virus. Aquaculture 495:359–365. https://doi.org/10.1016/j.aquaculture.2018.06.020
11. Mariot LV, Bolívar N, Coelho JDR, Goncalves P, Colombo SM, do Nascimento FV et al (2021) Diets supplemented with carrageenan increase the resistance of the Pacific white shrimp to WSSV without changing its growth performance parameters. Aquaculture 545. https://doi.org/10.1016/j.aquaculture.2021.737172
12. Godlewska K, Michalak I, Tuhy L, Chojnacka K (2016) Plant growth biostimulants based on different methods of seaweed extraction with water. Biomed Res Int 2016:1. https://doi.org/10.1155/2016/5973760
13. Athukorala Y, Lee KW, Song C, Ahn CB, Shin TS, Cha YJ et al (2003) Potential antioxidant activity of marine red alga grateloupia filicina extracts. J Food Lipids 10:251–265. https://doi.org/10.1111/J.1745-4522.2003.TB00019.X
14. Thanigaivel S, Chandrasekaran N, Mukherjee A, Thomas J (2019) Protective efficacy of microencapsulated seaweed extracts for preventing Aeromonas infections in Oreochromis mossambicus. Comp Biochem Physiol Part C Toxicol Pharmacol 218:36–45. https://doi.org/10.1016/J.CBPC.2018.12.011
15. Thanigaivel S, Vidhya Hindu S, Vijayakumar S, Mukherjee A, Chandrasekaran N, Thomas J (2015) Differential solvent extraction of two seaweeds and their efficacy in controlling Aeromonas salmonicida infection in Oreochromis mossambicus: a novel therapeutic approach. Aquaculture 443:56–64. https://doi.org/10.1016/J.AQUACULTURE.2015.03.010

Chapter 18

Treating Bacterial Infections in Fishes and Shrimps Using Seaweed Extracts

S. Thanigaivel, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

The use of seaweeds in the treatment of bacterial infections can help to reduce antibiotic resistance. Use of brown and green seaweed extracts, in particular, promotes immunological protection against fish and shrimp. For 45 days, juveniles were given seaweed extracts in concentrations of 2.5 and 5 g kg-1 in conjunction with their meal. The extracts contained bioactive molecules that were both antioxidant and antibacterial in nature, making them very effective against infections. When compared to the other extracts, the ethanol and aqueous extracts of the seaweeds give good antibacterial effect against bacterial infections in fish, indicating that they were more effective. Using algal extracts to treat infections in aquaculture is a cost-effective and ecologically friendly method of disease management. Using these extracts as a prophylactic step could be beneficial.

Key words Seaweed extracts, Pathogenic infections, Fish and shrimp pathogen, Pathogenicity, Treatment

1 Introduction

Aquaculture has seen a surge in popularity in recent years, particularly in the production of fish and shrimp, and this is expected to continue. Since the rapid expansion of the fish and shrimp industries has resulted in increased production of both fish and shrimps as a result of the rapid development of aquaculture and the increasing demand for fish, the production of both fish and shrimps has increased [1]. Disease outbreaks are therefore more likely to occur as a result of this. When it comes to mass mortalities in aquaculture, infectious diseases produced by bacteria, viruses, and fungi are among the most common causes. Viruses cause huge losses in both farmed fish and shellfish production. There are a variety of effective treatment methods available for various epidemics. Farmers commonly employ antibiotics and/or pesticides to control

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_18,

dangerous organisms in their fields [2]. Drugs such as antibiotics and chemicals, on the other hand, have been used sparingly since they are expensive, nonbiodegradable, and highly biomagnified, and antibiotic resistance has been increasing in recent years. Additionally, unfavorable side effects, such as ramifications for human health and the health of nontarget species, have been observed in some instances. Pesticides and antibiotics are being used indiscriminately, which has created a heated debate among environmentalists and government organizations about whether to outright ban these products or allow them to be manufactured in a more environmentally friendly manner [3]. It has received a great deal of interest in the recent decade to study pathogen management in aquaculture and illness prevention using herbs and phytochemicals. Several highly bioactive secondary metabolites found in seaweeds, such as sterols, hormones, vitamin B complexes, and bio-membrane structure components, have been shown to contribute in the creation of novel functional aquaculture ingredients. Several of these compounds have been studied for their potential anticarcinogenic, antibacterial, and anti-inflammatory properties [4]. To improve the defenses of shrimp, several researchers have concentrated on the development of new bioactive chemicals derived from seaweeds and other natural products. Furthermore, the increased need for disease control strategies that are less harmful to the environment has encouraged specialists to seek different options. The primary objective is to determine how different seaweed extracts affected the growth, survival, and immune protection of shrimp and fish against bacterial infections [5].

2 Materials

2.1 Collection of Experimental Fishes [6]

- Healthy fingerlings.
- Glass container.
- Aerator.
- Fiberglass tank.
- Test tubes.

2.2 Isolation of Bacterial Pathogen [4]

- Isolation of microorganism from infected fishes.
- Culturing in lab condition.
- Biochemical confirmation.
- 16 s rRNA identification.
- Koch postulate.
- Pathogenicity in fishes.

2.3 Preparation of Bacterial Inoculum [4]

- Culture bacteria on selective medium for pathogenicity experiments.
- Incubated at 37 °C for 24 h.

3 Methods

3.1 Maintenance of Experimental Animals [7]

- Collect fingerling of fishes.
- Transport the fingerlings with proper aeration.

3.2 Collection and Processing of Seaweeds

- Collect seaweeds and shade dry it.
- Crush the powder and soak 5 g in 100 mL of respective solvents for 48 h.
- Extract it using water, ethanolic, and other desirable solvents.
- Filter the extract and store in vitro and in vivo.

3.3 Antibacterial Activity [8]

- Prepare Mueller Hinton agar and autoclave at 121 °C for 15 min.
- Pour the media into sterile plates.
- Perform agar well diffusion against pathogen.
- Dip the broth culture with a sterilized cotton swab.
- Remove the extra inoculum by squeezing the cotton swab on the sides of the test tubes.
- Swab the cultures onto the media.
- Make two 8-mm-diameter wells with well cutter on the agar plate.
- Add 0.5, 1, 1.5, 2, and 2.5 mg/mL of extract into the wells.
- Add respective solvents as a control in separate well.
- Allow the solution for 2 h to disperse.
- Incubate the plates for 24–48 h at 37 °C.
- Observe the zone of inhibition around the well.

3.4 Pathogenicity and Treatment of Seaweed Extracts [8]

Add the 1000 mL of water in a glass tank.

Keep three tanks.

Add five healthy fishes in all the tanks.

Inject 20 μL of bacterial pathogen (10^5) dilution to fishes kept in tanks.

Inject 20 μL of saline to fishes in one tank which serve as control for pathogenicity.

- Inject 25 μL of respective extracts by intramuscular injection for treatment.
- Inject saline into one set of fishes for control treatment [4, 9].

References

1. Mondal H, Chandrasekaran N, Mukherjee A, Thomas J (2021) Viral infections in cultured fish and shrimps: current status and treatment methods. Aquac Int 2021:1–36. https://doi.org/10.1007/S10499-021-00795-2
2. Balboa EM, Conde E, Moure A, Falqué E, Domínguez H (2013) In vitro antioxidant properties of crude extracts and compounds from brown algae. Food Chem 138:1764–1785
3. Balasubramanian G, Sarathi M, Rajesh S, Kumar, Hameed ASS (2007) Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. Aquaculture 263:15–19
4. Thanigaivel S, Vidhya Hindu S, Vijayakumar S, Mukherjee A, Chandrasekaran N, Thomas J (2015) Differential solvent extraction of two seaweeds and their efficacy in controlling Aeromonas salmonicida infection in Oreochromis mossambicus: a novel therapeutic approach. Aquaculture 443:56–64. https://doi.org/10.1016/J.AQUACULTURE.2015.03.010
5. Chojnacka K (2012) Biologically active compounds in seaweed extracts – the prospects for the application. Open Conf Proc J 3:20–28. https://doi.org/10.2174/1876326X01203020020
6. Thanigaivel S, Vijayakumar S, Gopinath S, Mukherjee A, Chandrasekaran N, Thomas J (2015) In vivo and in vitro antimicrobial activity of Azadirachta indica (Lin) against Citrobacter freundii isolated from naturally infected tilapia (Oreochromis mossambicus). Aquaculture 437:252–255. https://doi.org/10.1016/J.AQUACULTURE.2014.12.008
7. Thanigaivel S, Thomas J, Vickram AS, Gulothungan G, Nanmaran R, Jenila Rani D (2021) Antioxidant and antibacterial efficacy of Chaetomorpha linum and its toxicological evaluation for the prophylactic treatment against Pseudomonas aeruginosa infection in Labeo rohita. https://doi.org/10.1080/10454438.2021.1961966
8. Aftabuddin S, Abdul M, Siddique M, Habib A, Akter S, Hossen S et al (2021) Effects of seaweeds extract on growth, survival, antibacterial activities, and immune responses of Penaeus monodon against Vibrio parahaemolyticus. https://doi.org/10.1080/1828051X.2021.1878943
9. Thanigaivel S, Chandrasekaran N, Mukherjee A, Thomas J (2019) Protective efficacy of microencapsulated seaweed extracts for preventing Aeromonas infections in Oreochromis mossambicus. Comp Biochem Physiol Part C Toxicol Pharmacol 218:36–45. https://doi.org/10.1016/J.CBPC.2018.12.011

Chapter 19

Preparation and Treatment of Seaweed Encapsulated Pellet Feed in Fisheries Aquaculture

S. Thanigaivel, John Thomas, Natrajan Chandrasekaran, and Amitava Mukherjee

Abstract

Aquaculture is one of the most effective methods of increasing world fish production. Disease outbreaks are a significant problem in the aquaculture industry. Using chemotherapeutic approaches, disease outbreaks have been treated and averted in the past. The usage of chemicals has detrimental consequences for the environment as well as for the health of humans. Antibiotic resistance occurs as a result of repeated usage of antibiotics over an extended period of time. The use of natural products such as medicinal plants, marine algae, herbs, and their extracted compounds in the management of disease in fish and prawns is now being explored. Single ingredients, combinations of two different compounds, and feed additives can all be employed. The compounds produced by the extracts are more effective when supplied as encapsulated beads.

Key words Seaweeds, Microencapsulation, Fish feed, Antibacterial compounds, Fish pathogen, Disease resistance

1 Introduction

Aquaculture provides protein supplements in the form of fish meal, which is produced in large quantities. Aquiculture includes the production of "food fish," which are vertebrates and invertebrates that are intended for human consumption, as well as the production of non-food items such as pearls, seashells, and decorative items [1]. There have been an enormous number of cultivated aquatic plant species, such as seaweed, and cultivable critters produced in recent years. Apart from that, almost 43 million tons of fish have been used as food sources for marine algae, which has a range of applications. Farm-raised fish account for the majority of primary production [2]. Fish residues account for the vast bulk of the world's total output of fish meal. International commerce in fisheries and export of fisheries products is a significant economic

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_19,

activity in developing nations, accounting for over 10% of total agricultural transportation in 2007. During the preceding five decades, global fish production has expanded tremendously [3]. In order to ensure long-term aquaculture productivity, the use of biobased natural products in aquatic animal health and disease management has become increasingly vital. Because of environmentally friendly techniques, this was made possible. These biobased natural products can be used to replace synthetic chemicals in the environment. Environmentally friendly processes are employed in the creation of such natural items, which aids in the solution of environmental problems created by synthetic chemicals [4]. It is necessary to compactly anchor the components using a polysaccharide including sodium caseinate and sodium alginate biopolymer matrix. Encapsulating bioactive chemicals in this manner is a novel approach of preventing biological degradation and oxidation of the molecules [5]. The bioactive characteristics of microencapsulated products are preserved for a longer period of time. The active chemicals isolated from seaweed extracts, which were chosen for their antibacterial and antioxidant capabilities, were investigated in the current study to determine their effects [6, 7]. They were microencapsulated in a biopolymer system in order to examine their antibacterial properties and growth-stimulating factors against diseases that affect fish and shrimp, with the goal of improving health and disease resistance in the animals [8, 9]. This development of a bioeffective diet supplement for use in fish feed is very effective [10].

2 Materials

2.1 Collection of Seaweeds and Experimental Fishes [11]

- Marine algae—brown and green seaweeds.
- Distilled water.
- Plastic containers and jars.
- Plastic trays.
- Petri dish.
- Mixer grinder.
- Filtering funnel.
- Conical flask.
- Healthy fingerlings.
- Glass container.
- Aerator.
- Fiberglass tank.
- Test tubes.

2.2 Extraction of Active Compounds [10, 12]

- Crude extracts of seaweeds.
- Column chromatography.
- Silica gel.
- Solvents.
- Blotting papers.

3 Methods

3.1 Preparation of Bioactive Compounds [10]

- Collect seaweeds and shade dry it.
- Powder the samples.
- Add 5 g of seaweeds with 100 mL of ethanol.
- Keep in shaker for 24 h.
- Take a glass column.
- Pack silica gel column (2 × 25 cm) with cotton wool.
- Add mobile phase solvent till it reach cotton wool.
- Add the extract into the column.
- Collect each fractions separately.

3.2 Encapsulation of Active Compounds [10]

- Mix sodium caseinate and xanthan gum with 1% glycerol and Milli-Q water.
- Add the purified bioactive compounds to the biopolymer matrix and mixed together.
- Perform mixing in magnetic stirrer.
- Keep for 24 h.
- Drop the biopolymer matrix into 1 N HCL using 5 mL syringe.
- Observed for bead formation.
- Filter the HCL using muslin cloth.
- Collect the beads and wash three times in Milli-Q water.
- Dry the beads over night at room temp.

3.3 Feed Treatment [10]

- Add the 1000 mL of water in a glass tank.
- Keep three tanks.
- Add five healthy fishes in all the tanks.
- Inject 20 μL of bacterial pathogen (10^5) dilution to fishes in two tanks.
- Inject 20 μL of saline to fishes in other tank.

- In one tank (fish injected with pathogen), add 1 g of beads (treated).
- In other two tanks, add normal fish feed (control).
- Observe the results and compare between control and treated.

References

1. Ma Q, Li LY, Le JY, Lu DL, Qiao F, Zhang ML et al (2018) Dietary microencapsulated oil improves immune function and intestinal health in Nile tilapia fed with high-fat diet. Aquaculture 496:19–29. https://doi.org/10.1016/J.AQUACULTURE.2018.06.080
2. Van Doan H, Hoseinifar SH, Faggio C, Chitmanat C, Mai NT, Jaturasitha S et al (2018) Effects of corncob derived xylooligosaccharide on innate immune response, disease resistance, and growth performance in Nile tilapia (Oreochromis niloticus) fingerlings. Aquaculture 495:786–793. https://doi.org/10.1016/j.aquaculture.2018.06.068
3. Padmaja R, Pramanik S, Pingali P, Bantilan C, Kavitha K (2019) Understanding nutritional outcomes through gendered analysis of time-use patterns in semi-arid India. Glob Food Sec 23:49–63. https://doi.org/10.1016/j.gfs.2019.04.001
4. Lawlor JB, Gaudette N, Dickson T, House JD (2010) Fatty acid profile and sensory characteristics of table eggs from laying hens fed diets containing microencapsulated fish oil. Anim Feed Sci Technol 156:97–103. https://doi.org/10.1016/J.ANIFEEDSCI.2010.01.003
5. Magowan E, McCann MEE, Beattie VE, McCracken KJ, Henry W, Smyth S et al (2010) The effect of the method of dietary oil addition to pelleted diets on performance and nutrient digestibility in finishing pigs. Anim Feed Sci Technol 156:89–96. https://doi.org/10.1016/j.anifeedsci.2010.01.002
6. Linke A, Linke T, Kohlus R (2020) Contribution of the internal and external oxygen to the oxidation of microencapsulated fish oil. Eur J Lipid Sci Technol 122:1900381. https://doi.org/10.1002/EJLT.201900381
7. Thanigaivel S, Vickram S, Saranya V, Ali H, Alarifi S, Kumar Reddy Modigunta J et al (2022) Seaweed polysaccharide mediated synthesis of silver nanoparticles and its enhanced disease resistance in Oreochromis mossambicus. J King Saud Univ Sci 34:101771. https://doi.org/10.1016/J.JKSUS.2021.101771
8. Willer DF, Aldridge DC (2019) Microencapsulated diets to improve bivalve shellfish aquaculture for global food security. Glob Food Sec 23:64–73. https://doi.org/10.1016/J.GFS.2019.04.007
9. Jones DA, Holland DL, Jabborie S (1984) Current status of microencapsulated diets for aquaculture. Microencapsul Artif Cells:275–288. https://doi.org/10.1007/978-1-4612-5182-8_32
10. Thanigaivel S, Chandrasekaran N, Mukherjee A, Thomas J (2019) Protective efficacy of microencapsulated seaweed extracts for preventing Aeromonas infections in Oreochromis mossambicus. Comp Biochem Physiol Part C Toxicol Pharmacol 218:36–45. https://doi.org/10.1016/J.CBPC.2018.12.011
11. Peixoto MJ, Ferraz R, Magnoni LJ, Pereira R, Gonçalves JF, Calduch-Giner J et al (2019) Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (Dicentrarchus labrax) subjected to bacterial infection. Sci Rep 9:1–12. https://doi.org/10.1038/s41598-019-52693-6
12. Xu S, Zhang L, Wu Q, Liu X, Wang S, You C et al (2011) Evaluation of dried seaweed Gracilaria lemaneiformis as an ingredient in diets for teleost fish Siganus canaliculatus. Aquac Int 19(5):1007–1018. https://doi.org/10.1007/S10499-011-9418-Z

Chapter 20

Treatment Using Seaweeds in Fishes and Shrimp by In Vivo Method

R. Bharath, K. Karthikeyan, R. Vidya, and R. Sudhakaran

Abstract

Disease outbreak is a serious issue in aquaculture sector, influencing the development of fishes and shrimp. The emergence of pathogens in shrimp and fish has caused significant economic impairment and had devastating impact on the aquaculture industry. However, there are no efficient therapeutic methods available for control of diseases. In order to control disease condition in shrimp and fishes, the use of medicinal plants, seaweeds, and their secondary metabolites has gained importance. Dietary supplementation with seaweeds has the ability to increase immunological response and physiological function. Here, we describe how the bioactive compound from seaweed is isolated and used as feed for aquatic animals.

Key words Seaweed extracts, Animals, Immune-response, Antioxidant

1 Introduction

Seaweeds or marine algae are marine aquatic plants that inhabit the coastal regions of oceans and seas [1]. These are macroscopic algae that are found attached to the solid bottom of rocks, shells, and other plant material. It constitutes about 6000 species with a great diversity of forms and sizes and only 5% of it is being used. Seaweeds have a great source of bioactive compounds and produce secondary metabolites that exhibit a wide range of biological function such as antibacterial [2], antifungal, antiviral, anti-inflammatory, nematocidal, and anticoagulant [3, 4]. Based on their pigmentation, they are divided into three categories, such as Rhodophyta (red algae), Chlorophyta (green algae), and Phaeophyta (brown algae). The demand for seaweed usage has increased in the last decade.

Aquaculture is the fastest-growing animal production industry. Shrimp and fish are one of the most traded aquaculture products, accounting for one of the largest groups of exported species in terms of price. Diseases are one of the key impediments in

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_20,

aquaculture development, costing the business more than $6 billion each year. The necessity of reducing chemical use is an important concern because the application of various chemotherapeutics including antibiotics has a deleterious influence on immune system of fish and shrimp [5]. To control the diseases in aquatic animals, several immunization strategies including DNA vaccines and recombinant vaccines were used. The oral route of administration (feed) is the most prominent route of delivery, but the infected animals may lose their appetites and fail to adequately ingest or digest their feed. This eventually leads to the release of antibiotics into the environment via feces. However, the development of vaccinations is more concerned with safety than effectiveness. The increased pressure to phase out certain traditional disease management measures is prompting farmers to look for non-harmful alternatives to treat and prevent disease outbreaks. In recent years, the interest toward seaweeds and their extract has increased due to their ability to boost growth, immunological response, and diseases resistance in aquatic animals. Seaweed exhibits great source of antimicrobial activity, but the amount of efficacy differs by species. Compared to other antibiotics and chemical drugs, the seaweed extracts are cost-effective and are easily biodegradable, and they easily degrade in natural aquaculture systems. The extract obtained can be either a single compound or a mixture of two different compounds. The delivery of compounds obtained from the extracts was shown to be more effective when delivered in the form of encapsulated beads [6].

2 Materials

2.1 Collection of Seaweeds

- Seaweeds.
- Grinder.
- Airtight container.

2.2 Preparation of Seaweed Extract

- Seaweed powder.
- Petroleum ether.
- Acetone.
- Chloroform.
- Methanol.
- Incubator.
- Whatman no. 1 filter paper.
- Rotary vacuum evaporator.

2.2.1 In Vitro Antibacterial Activity

- Sterile cotton swab.
- Sterile well cutter.
- Agar plate.
- Seaweed extract.

2.3 Treatment Using Seaweed Extract

- NTE buffer.
- Seaweed extract.

2.3.1 Bioassay

- Fish/shrimp.

2.4 Treatment Using Pellet Feed

2.4.1 Preparation of Seaweed Diet

- Fish meal.
- Groundnut oil cake.
- Soybean powder.
- Wheat bran.
- Vitamins.
- Mineral mix.
- Binder.
- Seaweed active fraction (SAF).

2.4.2 Feeding Trail

- Pellet feed.
- Fish/shrimp.

2.5 Determination of Immune Parameters After Bioactive Compound Administration

- 1 mL needle.
- 1.5 mL microcentrifuge tube.
- Anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA at pH 7.5).

2.5.1 Blood Sample Analysis

3 Methods

3.1 Collection of Seaweeds

- Collect fresh seaweeds and remove impurities and salts present on the surface using tap water.
- Shade dry the sample for 72 h.
- Prepare dried seaweeds into fine powder and store in an airtight container for further experiments.

3.2 Preparation of Seaweed Extract

- Weigh 10 g of powdered seaweed and extract it using different solvents such as petroleum ether, acetone, chloroform, methanol, and water (1:100 w/v).
- Keep it in shaker incubator for 24 h at 37 °C [7].

- Then filter the extract using Whatman no. 1 filter paper and evaporate via rotary vacuum evaporator.
- To calculate the sample's dry yield, determine the dried yield of the seaweed extract produced using differential extraction procedure.

3.3 In Vitro Antibacterial Activity

- To determine the antibacterial activity of seaweed extract, agar well diffusion technique can be performed.
- A sterile cotton swab was used to disseminate an inoculum of the bacterial pathogen at 10^8 cfu/mL on top of the solidified agar plate.
- Then by using a sterile well cutter, punch 8-mm-diameter well into the agar plate.
- Add various concentrations of seaweed extract and allow it to diffuse for 2 h.
- Then incubate the plate at 37 °C for 24 h.
- The antibacterial activity will be determined with the highest zone of inhibition around the well [8, 9].

3.4 Treatment Using Seaweed Extract

3.4.1 Bioassay

- The antibacterial and antiviral activity of seaweed extract in vivo can be examined by intramuscular injection.
- Divide the animals into three groups and conduct the study in triplicates.
- Group 1 was injected with NTE buffer served as negative control; group 2 was injected with 100 µL (5 µL of pathogen and 95 µL of NTE buffer) pathogen which served as positive control; and group 3 was injected with 100 µL (5 µL of pathogen, 20 µL of extract, and 75 µL of NTE buffer) pathogen challenged with 1 mg/mL extract.
- Feed the animals twice a day using commercially available feed [10].
- The CM% can be calculated by the total number of animals in the control group by the total number of animals in experimental groups multiplied by 100 [11].

3.4.2 GC–MS Analysis of Seaweed Extract for Identification of Bioactive Compounds

- To determine the chemical composition of seaweed extracts, as well as the presence of bioactive natural compounds, GC–MS can be used [12].

3.4.3 FTIR Analysis for Identification of a Functional Group

- To identify functional groups, present in the seaweed extract, FTIR spectroscopy can be performed [13].

3.5 Treatment Using Pellet Feed

3.5.1 Preparation of Seaweed Diet

- The pellet diet contains 56% fish meal, 20.7% groundnut oil cake, 11% soybean powder, 6% wheat bran, and 2% vitamins and mineral mix, 2% cod liver oil, and 2% binder [14].
- The solvent extract of the seaweed active fraction (SAF) should be included individually into the test at a different concentration mixed with the basal ingredients at 100, 200, 300, and 400 mg kg^{-1}.
- Using manual feed pelletizer, pelletized the feed through a sieve (2 mm).
- In control diet, seaweed active fraction is not added.
- The feeds can then be air-dried at 60 °C for 18 h before being packed individually in the appropriate vessel.

3.5.2 Feeding Trail

- Inject the pathogen intramuscularly (IM) into the shrimp or fish from the experimental and control groups [15].
- During the challenge experiment, feed all the animals with relevant experimental diets two times a day.
- Perform the study in triplicates and for negative control normal feed should be given.
- The unfed and fecal matter must be removed daily by water exchange.

3.5.3 Growth Performance

- At the end of the experiment period, estimate the growth parameters by dividing total animal weight in each net enclosure by the number of animals [16].
- The following variables can be estimated:

  Weight gain (WG) (%) $= 100 \times (W_f - W_i)/W_i$.

  Specific growth rate (SGR) (% day^{-1}) $= 100 \times (\ln W_f - \ln W_i)/t$.

  Survival (%) $= 100 \times N_t/N_o$.

  Feed conversion ratio (FCR) = dry feed intake$/(W_t - W_o)$.

  Protein efficiency ratio (PER) $= 100 \times (W_t - W_o)/(I \times C_{Nf})$.

3.6 Determination of Immune Parameters After Bioactive Compound Administration

3.6.1 Blood Sample Analysis

- Collect blood or hemolymph from the randomly selected animals in each group at the end of the experiment.
- Collect 0.5 mL of blood from each fish through cardiac vein puncture with 1 mL needle [17].
- In shrimp withdraw 0.5 mL of hemolymph from the ventral sinus cavity of each shrimp.
- Then add the samples to a tube containing 0.5 mL anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA at pH 7.5).

- Measure THC (total hemocyte count), TPP (total plasma protein), phenoloxidase activity (PO), peroxidase activity (POD), and superoxide dismutase activity (SOD), and store in −20 °C for further study.

3.6.2 RT-PCR Analysis

- Total RNA was extracted and was converted into cDNA by reverse transcription method [18].
- The mRNA expression of immune gene in animals fed with control and experimental diets can be analyzed using real-time PCR.

3.6.3 Histological Analysis

- For comparative analysis of healthy, infected, and treated animals, different organs, such as gills, hepatopancreas, muscle, etc., can be collected by dissection and can be used for examination [19].

References

1. Leupp JL, Caton JS, Soto-Navarro SA, Lardy GP (2005) Effects of cooked molasses blocks and fermentation extract or brown seaweed meal inclusion on intake, digestion, and microbial efficiency in steers fed low-quality hay. J Anim Sci 83:2938–2945. https://doi.org/10.2527/2005.83122938X
2. Singh AP, Chaudhary BR (2010) Preliminary phycochemical analysis and in vitro antibacterial screening of pithophora oedogonia (Mont.) Wittrock – a freshwater green alga forming mats in the water bodies. J Algal Biomass Utln 1:33–41
3. Caijiao C, Leshan H, Mengke Y, Lei S, Miansong Z, Yaping S, Changheng L, Xinfeng B, Xue L, Xin L, Airong J (2021) Comparative studies on antioxidant, angiotensin-converting enzyme inhibitory and anticoagulant activities of the methanol extracts from two brown algae (Sargassum horneri and Sargassum thunbergii). Russ J Mar Biol 47(5):380–387. https://doi.org/10.1134/S1063074021050035
4. de Almeida CLF, Falcão H d S, Lima GR d M, Montenegro C d A, Lira NS, de Athayde-Filho PF, Rodrigues LC, de Souza MFV, Barbosa-Filho JM, Batista LM (2011) Bioactivities from Marine Algae of the Genus Gracilaria. Int J Mol Sci 12:4550–4573. https://doi.org/10.3390/IJMS12074550
5. Park JM, Ruess L, O'Connor SC, Hussain F, Oshiro DY, Person DA (2004) Internet consultations from a remote Pacific Island: impact of digitized radiologic images on referral decisions. J Digit Imaging 17(4):253–257. https://doi.org/10.1007/S10278-004-1022-6
6. Elshobary M, El-Shenody R, Ashour M, Zabed H (n.d.) Antimicrobial and antioxidant characterization of bioactive components from Chlorococcum minutum. Food Biosci. Bioscience, Undefined 2020. https://www.sciencedirect.com/science/article/pii/S2212429219306078. Accessed 30 Nov 2021
7. Zakaria N, Ibrahim D, Sulaiman S, Supardy A (n.d.) Assessment of antioxidant activity, total phenolic content and in-vitro toxicity of Malaysian red seaweed, Acanthophora spicifera. Undefined 2011. Researchgate.Net. https://www.researchgate.net/profile/Afifah-Supardy/publication/286395213_Assessment_of_antioxidant_activity_total_phenolic_content_and_invitro_toxicity_of_Malaysian_red_seaweed_Acanthophora_spicifera/links/572bd0fc08aef7c7e2c6b924/Assessment-of-antioxidant-activity-total-phenolic-content-and-invitro-toxicity-of-Malaysian-red-seaweed-Acanthophora-spicifera.pdf. Accessed 30 Nov 2021
8. Raghunathan G, Dhayanithi NB, Ajith Kumar TT, Kumaresan S (n.d.) Evaluation of antibacterial activity and immunostimulant of red seaweed Chondrococcus hornemannii (Kuetzing, 1847) against marine ornamental fish.

Undefined 2014. Researchgate.Net. https://www.researchgate.net/profile/Dr-Nb-Dhayanithi/publication/263651504_Evaluation_of_antibacterial_activity_and_immunostimulant_of_red_seaweed_Chondrococcus_hornemanni_Kuetzing1847_against_marine_ornamental_fish_pathogens/links/00b4953b68fdf2bc36000000/Evaluation-of-antibacterial-activity-and-immunostimulant-of-red-seaweed-Chondrococcus-hornemanni-Kuetzing-1847-against-marine-ornamental-fish-pathogens.pdf. Accessed 30 Nov 2021

9. Fatima R, Nilofer PS, Karthikeyan K, Vidya R, Itami T, Sudhakaran R (2022) Enhancement of immune response and resistance to Vibrio parahaemolyticus in tilapia fish (Oreochromis mossambicus) by dietary supplementation of Portieria hornemannii. Aquaculture 547: 737448. https://doi.org/10.1016/J.AQUACULTURE.2021.737448
10. Thanigaivel S, Vidhya Hindu S, Vijayakumar S, Mukherjee A, Chandrasekaran N, Thomas J (2015) Differential solvent extraction of two seaweeds and their efficacy in controlling Aeromonas salmonicida infection in Oreochromis mossambicus: a novel therapeutic approach. Aquaculture 443:56–64. https://doi.org/10.1016/J.AQUACULTURE.2015.03.010
11. Kubilay A, Altun S, Ulukoy S, Ekici S, Diler O (2008) Immunization of rainbow trout (Oncorhynchus mykiss) against Lactococcus garvieae using vaccine mixtures. Isr J Aquacult Bamidgeh 60:268–273. http://evols.library.manoa.hawaii.edu/handle/10524/19264 Accessed 30 Nov 2021
12. Thomas J, Jerobin J, Seelan TSJ, Thanigaivel S, Vijayakumar S, Mukherjee A, Chandrasekaran N (2013) Studies on pathogenecity of Aeromonas salmonicida in catfish Clarias batrachus and control measures by neem nanoemulsion. Aquaculture 396–399:71–75. https://doi.org/10.1016/J.AQUACULTURE.2013.02.024
13. Wongprasert K, Rudtanatip T, Praiboon J (2014) Immunostimulatory activity of sulfated galactans isolated from the red seaweed Gracilaria fisheri and development of resistance against white spot syndrome virus (WSSV) in shrimp. Fish Shellfish Immunol 36:52–60. https://doi.org/10.1016/J.FSI.2013.10.010
14. Abdelhamid AF, Ayoub HF, Abd El-Gawad EA, Abdelghany MF, Abdel-Tawwab M (2021) Potential effects of dietary seaweeds mixture on the growth performance, antioxidant status, immunity response, and resistance of striped catfish (Pangasianodon hypophthalmus) against Aeromonas hydrophila infection. Fish Shellfish Immunol 119:76–83. https://doi.org/10.1016/J.FSI.2021.09.043
15. Esquer-Miranda E, Nieves-Soto M, Rivas-Vega ME, Miranda-Baeza A, Piña-Valdez P (2016) Effects of methanolic macroalgae extracts from Caulerpa sertularioides and Ulva lactuca on Litopenaeus vannamei survival in the presence of vibrio bacteria. Fish Shellfish Immunol 51: 346–350. https://doi.org/10.1016/J.FSI.2016.02.028
16. Arizo MAM, Simeon EC, Layosa MJT, Mortel RMM, Pineda CMB, Lim JJE, Maningas MBB (2015) Crude fucoidan from Sargassum polycystum stimulates growth and immune response of Macrobrachium rosenbergii against white spot syndrome virus (WSSV). AACL Bioflux 8. http://www.bioflux.com.ro/aacl. Accessed 5 Jan 2022
17. Rama Nisha P, Elezabeth Mary A, Uthayasiva M, Arularasan S (2014) Seaweed Ulva reticulata a potential feed supplement for growth, colouration and disease resistance in fresh water ornamental gold fish, Carassius auratus. J Aquac Res Development 5(254):2
18. (PDF) Confirmation of anti-WSSV activity from Red Algae Hypnae spinella in freshwater crab Paratelphusa hydrodomous (n.d.). https://www.researchgate.net/publication/283509025_Confirmation_of_Anti-WSSV_activity_from_Red_Algae_Hypnae_spinella_in_freshwater_crab_Paratelphusa_hydrodomous. Accessed 5 Jan 2022
19. Thanigaivel S, Vijayakumar S, Mukherjee A, Chandrasekaran N, Thomas J (2014) Antioxidant and antibacterial activity of Chaetomorpha antennina against shrimp pathogen Vibrio parahaemolyticus. Aquaculture 433:467–475. https://doi.org/10.1016/J.AQUACULTURE.2014.07.003

Part IV

Treatment Using Medicinal Plants

Chapter 21

Treatment Using Medicinal Plants in Fish and Shrimp

Vernita Priya, A. T. Manishkumar, K. Karthikeyan, and R. Sudhakaran

Abstract

Fish, like other animals, are susceptible to a variety of diseases. The disease is a major factor in fish mortality, especially in young fish. Viral infections, bacterial infections, fungal infections, and other pathogens can cause fish illnesses. Antimicrobial drug use in aquaculture could result in pathogenic bacteria developing resistance. Alternatives to antibiotics have emerged in recent years and medicinal plants are one of the options provided. Secondary metabolites and phytochemical substances found in these plants exhibit antimicrobial properties in fish. The fact that they are native to the area gives them an edge, and the majority of these plants are not harmful to humans and fish. This chapter summarizes the methods of treatment using plant extracts as a long-term and successful alternative to chemical treatments in fish farming.

Key words Medicinal plants, Immunostimulants, Antibacterial, Antiviral, Antifungal, Antiparasitic

1 Background

The fastest-growing food industry is aquaculture; however, global demand will require a 50% increase in production by 2050. Every year, aquaculture expands at a faster rate, and the most prevalent sickness among pond caretakers is a fish disease, which spreads quickly and causes uncontrollable loss and industry shutdown. The crab sector, among the various aspects of aquaculture, has grown dramatically in recent years as a result of global market demand for crustaceans [1].

Plants are increasingly being employed as a treatment for a variety of ailments, particularly those in which synthetic medications induce negative effects. The prevalence of diseases has increased in aquaculture production and has gotten more intensive, resulting in large economic losses. Vaccinations are costly for a large number of fish growers, and their drawback of only being effective against a single infection. Antimicrobials and other veterinary med-

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_21,

ications are commonly used as prophylactics (to prevent infections before they arise), therapeutics (to treat sick animals), and growth promoters in fish food, baths, and injections [2].

Traditional medicine is based mostly on medicinal herbs, which are consumed by roughly 3.3 billion people in underdeveloped countries. Due to the presence of active components such as phenolics, flavonoids, and others, plant extracts in aquaculture may also show various activities such as stress reduction, appetite stimulation, growth promotion, enhancement of immunostimulant, antipathogen activity, and maturation of culture species. Due to their antioxidant and antibacterial characteristics, herbal extracts offer crucial properties such as disease control. Plant extracts are known to have various modes of action due to their complexity [3].

In aquaculture, bacterial illness is a severe problem, and antibiotics are occasionally used to treat it. Bacteria are the pathogenic agents with the greatest economic impact. Bacterial illnesses kill a lot of fish, both in captivity and in the wild, all over the world. Antibiotic use regularly can lead to bacterial resistance and undesirable residues in aquaculture products and the environment. Bacterial infection of fish and its products may have an impact on human health, either directly by causing illness or indirectly via antimicrobial drug residues' leftover from treating infections in fish that affect humans. Resistant bacterial strains may harm fish illness treatment and the environment of fish farms [3].

The breeding of marine fish is quite profitable in India. The rise of viral-caused infectious illness, which is affecting many high-value fish species and causes massive economic loss, is one of the biggest risks to the aquaculture business. Plant-based natural compounds are an important source for finding effective antiviral medicines. The most significant technique for testing the efficacy of an antiviral treatment is animal cell culture. For viral diagnostics, several fish cell lines have recently been produced [4].

In most cases, fungi infect fish as a result of another factor or pathogen, such as poor water quality, poor condition, trauma (rough handling or aggression), bacterial illness, or parasites. Fungi can be exterior or inside, and they can spread throughout the body. Fungi can wreak havoc on reproduction by infecting fertilized eggs in spawns, for example. In poorly stored feeds, certain species of fungi can thrive and create mycotoxins. Freshwater and brackish water fish are most commonly infected by water molds, which are the most prevalent of all fungal infections [5]. Fungal agents have gotten a lot of attention over the last decade, especially in wild fish populations.

2 Soxhlet Method

2.1 Materials

- Plant material.
- Flask.
- Soxhlet extractor.
- Glass wool.

2.2 Methods

- Fresh plant material or dried plant material is taken.
- The amount of plant material utilized should be enough to fill the porous cellulose thimble.
- The solvent (ethanol) transferred to a round-bottom flask.
- Inside Soxhlet extractor, crushed plant stuff is placed.
- Glass wool is taken and placed on the side arm of the Soxhlet extractor. The isomantle heats the solvent, which starts to evaporate as it passes through the device to the condenser.
- The solvent reaches the syphon and pours back into the flask, and the cycle is repeated for 16 h.
- Following the completion of the process, rotary evaporator is used to evaporate the ethanol, leaving a small amount of extracted plant material in the glass bottom flask (approximately 2–3 mL) [6].

3 Digestion

3.1 Materials

- Plant material.
- Water bath/oven.

3.2 Methods

- This is a method of extraction that uses mild heat during the extraction process.
- The extracted solvent is placed in a clean container, with the powdered drug material.
- At a temperature of about 50° C, the mixture is placed in water bath.
- Throughout the extraction procedure, heat is needed to reduce the viscosity of the extraction solvent and improve the removal of secondary metabolites.
- Plant materials which are easily soluble are used.
- Filtration and decantation is performed to separate the extract [7].

4 Plant Dye Extraction

4.1 Materials

- Plant material.
- Cheese cloth/cotton wool/paper filter.

4.2 Methods

- Different plant parts which are used to manufacture dye are taken.
- Plant part is collected and dried in the shade or in the sun before extraction.
- Homogenize using manual or electric grinding equipment to break it down into very small pieces or powder.
- Optimal extraction conditions determined by varying extraction parameters such as solvent type, extraction time, plant material to solvent ratio, temperature, and pH, which are all dependent on the properties of specific dye components.
- Following extraction, filter the extract filters using cheesecloth, cotton wool, or a paper filter [7].

4.3 Source of Medicinal Plant

Medicinal plants with different parts, like leaves, rhizome, roots, fruit, bark, seed, and bulb, are used to extract different secondary metabolites and phytochemical compounds, like alkaloids, flavonoids, and tannins [8].

4.4 Preparation of Fish Feed Formulations

- The dried feed (with a final moisture level of 6–10%), semi-moist (with 35–40%), or wet (with a moisture content of 50–70%).
- Depending on the fish's feeding requirements, the pellets will be modified to sink or float [8].
- Feed ingredients mixed for 5 min before adding 200 mL kg^{-1} of water and mixing for another 5 min.
- The mixture is then formed into a ball-like structure and autoclave for 45 min.
- After autoclaving, the herbal ingredients should be thoroughly mixed [9].
- The cylindrical dried feed will be packed in plastic bags and stored in a cool, dark place.
- Fish feeding is done at a rate of 3% twice a day in feeding trials for 8 weeks. The edge of the fins will develop and destroy more tissue over time [10].

4.5 Administration of Medicinal Plants in Aquaculture

These techniques of administering medicinal herbs for preventing and treating various fish illnesses are stated to have no dangers of contaminating the environment or harming fish and humans.

- Medicinal herbs administered to fish and shellfish through injection (intramuscular or intraperitoneal), oral delivery, and immersion or bathing [11].
- The most common technique of administering medicinal plants is through oral administration. Bacterial infections can be treated by including them in fish diet.
- The therapeutic or preventive dose must be calculated using the medicinal plant and the number of fish swallowed [12].
- Bathing fish in various medicinal plant solutions can also yield positive results. Various plant extracts can treat bacterial and fungal infections by immersing fish in them. The therapeutic or preventive dose should be calculated using the medicinal plant and the number of fish swallowed.
- Intraperitoneal injection is the most effective way to deliver the appropriate dose. An injection quickly raises the antibacterial substance levels in the blood and tissues [12].
- Medicinal plants could be used alone or in combination with trace elements and probiotics to treat fish diseases [12].
- Baths are often used to treat ectoparasites. As a result, oral treatment is a good alternative for aquaculture, as medicinal herbs can improve fish health and disease resistance.

4.6 Bacterial Diseases of Fish and Shrimp

In aquaculture, bacterial illness is a severe problem, and antibiotics are occasionally used to treat it. Antibiotic use on a regular basis can lead to bacterial resistance and undesirable residues in aquaculture products and the environment.

5 Materials and Methods

5.1 Preparation of Herb Extracts

- *Eclipta alba* is used.
- To facilitate easy solubility of the herb extract in antibacterial testing, the crude extract is further producing as complex granules using polyvinylpyrrolidone (PVP) [13].

5.2 Antibacterial Tests

- Antibacterial testing is done using Tragen's agar plate dilution method.
- Guava extract will be made in a series of twofold dilutions providing values ranging from 0.625 to 10 mg/mL.
- To reduce the inoculum for the test plates, preculture in tryptic soy broth for 18 h at 30 °C. Two percent NaCl is added as a supplement.

- Before overlaying on the test plates, the precultured broth (0.1 mL) is mixed with 1 mL of the same broth.
- Antibacterial activity will be tested by seeing bacteria develop after incubation at 30 °C for 24 h [13].

5.3 Efficiency of Herbs

10 mg/mL of extracts from *E. alba* can completely inhibit all of the microorganisms tested (100%). One (8.3%) and ten (83.3%) of the studied strains can be suppressed by concentrations of 2.5 and 5 mg/mL, respectively. This plant can prevent *Macrobrachium rosenbergii* from *Aeromonas hydrophila* infections [13].

5.4 Viral Diseases of Fish and Shrimp

5.4.1 Preparation of Viral Inoculum

- Using sterile syringes, infected shrimp's hemolymph is drawn directly from the heart.
- Centrifuge the hemolymph at 3000*g* for 20 min at 4 °C.
- The supernatant will be filtered through a 0.4 m filter after being recentrifuged at 8000*g* for 30 min at 4 °C.
- Store the filtrate at 20 °C for infectivity testing [14].

5.4.2 Preparation of Plant Extracts

Medicinal plant *Cynodon dactylon* which is previously reported for antiviral activity can be used.

- The plant is dried and extraction is done separately with petroleum ether, benzene, diethyl ether, chloroform, ethyl acetate, methanol, ethanol, and distilled water using a Soxhlet device.
- Crude extract will be filtered using Whatman No. 1 filter paper before drying in a vacuum evaporator at 40 °C and 25–30 mm Hg.
- The crude extracts (100 mg) are mixed in 0.2 mL acetone before mixing with 0.8 mL distilled water at 1000 ppm.
- In the final test, polysorbate 80 (Tween 80) is used with the extract. Negative control extract is made up of a mixture of acetone and polysorbate 80 [14].

5.4.3 Determination of Antiviral Activity

- For the bioassay, a 30 μL of viral suspension, plant extract, and NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl, and 0.02 M EDTA, pH 7.4) is injected intramuscularly into shrimp or freshwater crabs (five animals per tank) (5 L viral suspension, 10 L plant extract with varying concentrations (100 or 150 mg/kg of animal body weight), and 15 L NTE buffer).
- A mixture of 25 μL NTE buffer and 5 μL virus suspension is used as positive control, which will be combined with plant extracts at varied doses.

- Before injection keep it at 29 °C for 3 h.
- The combination is delivered intramuscularly into experimental animals after 3 h.
- After infection, the experiment will be carried out for up to 30 days. Record the results [14].

References

1. Ghosh AK, Panda SK, Luyten W (2021) Anti-vibrio and immune-enhancing activity of medicinal plants in shrimp: a comprehensive review. Fish Shellfish Immunol 117:192–210. https://doi.org/10.1016/j.fsi.2021.08.006
2. Reverter M, Bontemps N, Lecchini D, Banaigs B, Sasal P (2014) Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. Aquaculture 433:50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048
3. Muniruzzaman M, Chowdhury MBR (2009) Sensitivity of fish pathogenic bacteria to various medicinal herbs. Bangladesh J Vet Med 2. https://doi.org/10.3329/bjvm.v2i1.1941
4. Krishnan K, Khanna VG, Hameed S (2010) Antiviral activity of dasyscyphin C extracted from Eclipta prostrata against fish nodavirus. J Antivir Antiretrovir 2(1):29–32. https://doi.org/10.4172/jaa.1000018
5. Yanong RPE (2003) Fungal diseases of fish. Vet Clin North Am Exot Anim Pract 6(2):377–400. https://doi.org/10.1016/S1094-9194(03)00005-7
6. Redfern J, Kinninmonth M, Burdass D, Verran J (2014) Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. J Microbiol Biol Educ 15(1):45–46. https://doi.org/10.1128/jmbe.v15i1.656
7. Abubakar AR, Haque M (2020) Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. J Pharm Bioallied Sci 12(1):1–10. https://doi.org/10.4103/jpbs.JPBS_175_19. Epub 2020 Jan 29. PMID: 32801594; PMCID: PMC7398001
8. Pandey G (2013) Feed formulation and feeding technology for fishes. Int Res J Pharm 4(3):23–30. https://doi.org/10.7897/2230-8407.04306
9. Sinha A, Mondal C, Santra S. Exploration of canal resources for development of fisheries in Sunderbans (W.B.) and Punjab. Aquaculture Asia, pp 3–8. www.ijltemas.in
10. Santra S, Sinha A, Mondal C (2017) Effect of herbal plant (Tulsi) against common disease in gold fish, Carassius auratus (Linn. 1758). International Journal of Latest Technology in Engineering, Management & Applied Science (IJLTEMAS) 6:29–31
11. Ramudu KR, Dash G (2013) A review on herbal drugs against harmful pathogens in aquaculture. Am J Drug Discov Dev 3(4):209–219
12. Stratev D, Zhelyazkov G, Noundou XS, Krause RWM (2018) Beneficial effects of medicinal plants in fish diseases. Aquacult Int 26(1):289–308. https://doi.org/10.1007/s10499-017-0219-x
13. Direkbusarakom S, Ezura Y, Yoshimizu M, Herunsalee A (1998) Efficacy of Thai traditional herb extracts against fish and shrimp pathogenic bacteria. Fish Pathol 33:437
14. Balasubramanian G, Sarathi M, Kumar SR, Hameed ASS (2007) Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. Aquaculture 263(1–4):15–19. https://doi.org/10.1016/j.aquaculture.2006.09.037

Chapter 22

Preparation of Feed and Characterization of Feed Supplemented with Phytocompounds

N. Chandra Mohana, A. M. Nethravathi, Raghu Ram Achar, K. M. Anil Kumar, and Jalahalli M. Siddesha

Abstract

Aquaculture has gained prominence globally in recent years particularly in fish and shrimp production. The increased surge of demand has led to a rapid increase in several aquaculture setup implementations. However, it also has raised the risks of disease outbreaks leading to mass mortality rates caused by bacteria, viruses, and fungi. Also, inappropriate use of antibiotics leading to resistance in microbial pathogens is an expanding threat. Hence, feed preparation and characterization using ethnomedicinal plants could offer relief for the present requisite in aquaculture. Feed formulation by analyzing nutritional needs supplemented with ethnomedicinal plant-based feeds would benefit aquaculture in encountering antibiotic resistance. The feed formulation using plant-based feeds would envision natural solutions for infections along with economical sustainability with low-cost feed for the growing aquaculture industry.

Key words Aquaculture, Feed, Medicinal plants, Antimicrobial, Phytocompounds

1 Introduction

In terms of species, agricultural methods, and environmental circumstances, aquaculture is the world's most diverse farming practice. Aquatic farming is a rapidly expanding agricultural sector for the production of high-protein meals with fish and shellfish being widely produced globally [1, 2]. Fish has long been recognized as a low-cost, high-quality protein source, with demand and consumption rising in several developing countries [3]. It is impossible to precisely calculate the total number of farmed species and the extent to which they are farmed; typically, reports that include such figures are limited and inaccurate.

The primary challenges for fish farmers are cultivating highly produced fish and disease-resistant cultured fish [4]. Also, the high cost of high-quality fish feed is one of the barriers to aquaculture development. With disease outbreaks increasing in lockstep with

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_22,

the expansion of intensive aquaculture, the need for low-cost fish feed with multifunction attributes is the need of the hour. The inappropriate use of antimicrobial drugs in fish aquaculture is worsening the global problem of antibiotic resistance [5]. The expanding demand for animal protein for human use has resulted in an alarming increase in the necessity for lifesaving medications in these modern animal production systems. Because of the emergence of antibiotic resistance in bacteria, which threatens to undo most of the medical progress made in the previous decades, the number of antimicrobials available for disease treatment is becoming increasingly limited, expensive, and, in some cases, unavailable. According to the findings of the study, the lack of aggressive disease control has harmed long-term fish output [6].

Antimicrobials have primarily been used in aquaculture for therapeutic and preventative purposes [7]. With antibiotics being completely banned as a feed additive in the European Union due to concerns that they may contribute to bacterial resistance or endanger human health via residues in animal by-products, there is a need for natural alternatives [8]. Natural resources, such as medicinal herbs, have been used to treat a wide range of human illnesses for thousands of years. Numerous active compounds with potential bioactivities have been identified.

As a result, there has been a surge in interest in using medicinal plants in aquaculture to provide safe and environmentally acceptable alternatives to antibiotics and chemical compounds, as well as to boost immune function and control fish diseases. Herbs, seeds, and spices are used as immunostimulants in a variety of forms, including crude, extracts, blended, and active chemicals, resulting in a significant improvement in fish immune systems, allowing them to avoid and control microbial illnesses. Plant segments such as seeds, roots, blooms, and leaves also displayed varying degrees of activity. As measured by increases in immunological parameters, medicinal plants were found to boost both cellular and humoral immune responses. When injected, immersed, or taken orally, medicinal plants have been shown to boost the immune system to varying degrees and quantities. However, determining the optimal dose to boost fish immune systems while avoiding immunosuppression is critical. Certain medicinal plants have been successfully used to replace the protein in fish meal as a low-cost source of protein. Medicinal plants can function as both an immunomodulator and a growth stimulant [9].

2 Materials

(i) Proteins (essential amino acids).

(ii) Carbohydrates (sugars, starch).

(iii) Lipids (fats, oils, fatty acids).
(iv) Vitamins.
(v) Minerals.
(vi) Medication: plant extracts/phytocompounds.
(vii) Aqueous and organic solvents.
(viii) Pelleting apparatus.

3 Methods

3.1 Preparation of Feed Supplemented with Phytocompounds

The controlling of microbial infections in aquaculture is traditionally done with the application of antimicrobial agents in aquaculture. In this regard, medicated feeds are being developed by using medicinal plants [5]. The medicinal plants and extracts with antibacterial, antiviral, and antifungal activity in fish and shrimps have been enlisted in Table 1, and the steps involved in feed preparation and characterization have been depicted in Fig. 1.

Aqueous Extract Preparation [10]

- Weigh and dissolve the plant material in 100 mL of water.
- The aqueous solution is heated in a water bath at 80 °C for 15 min.
- The mixture is then allowed to cool to room temperature before being centrifuged at 6000 rpm for 10 min.
- The supernatant solution's filtrate is collected and mixed with fish feed.

Alcoholic Extract Preparation [11]

- In 100 mL EtOH/MeOH, ground air-dried plant material is shaken.
- The alcoholic solution is heated for 48 h at 40 °C.
- Filter paper (Whatman No. 1/4) was used to filter the insoluble material, which was then evaporated to dryness at 40 °C under decreased pressure.
- Weigh the extract and combine it with fish feed.

Solid Fish Feed Preparation [12]

- Plant material is thoroughly washed, rinsed, and cut into small pieces.
- Plant material is weighed, and paste is obtained with aid of a homogenizer.

Table 1
Medicinal plants exhibiting antimicrobial activities in aquaculture

Name of plant	Name of microorganism
Vitis adnata (essential oil)	*Salmonella pullorum*
Cymbopogon citrates (oil)	*Shigella flexneri*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*
Chamaesyce hirta	*Shigella flexneri*
Ipomea fistulosa (ethanolic extract)	*Streptococcus faecalis*
Cabbage juice	*Staphylococcus aureus*
Anacardium occidentale	*Staphylococcus aureus*, *Serratia marcescens*
Bhallatakasava and Sukshma Triphala	*Clostridium titani*
Eucalyptus oil	*Mycobacterium avium*
Centella asiatica	*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Psendomonas cichorii*
Terminalia bellerica, *Garcinea gummigulla*, *Anisomeles malabarica*, *Aegle marmelos*, *Alangium saluifolium*, and *Zizyphus jujuba*	*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*
Azadirachta indica	*Mycobacterium tuberculosis*, *Streptomycin resistant strains*
Ocimum sanctum	*Escherichia coli*, *Bacillus anthracis*, *Mycobacterium tuberculosis*
Gloriosa superb (leaf extract)	*Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*
Triphala churna, Hareetaki churna and Dashmula churna	*Staphylococcus epidermidis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*
Moringa oleifera(leaf extract)	*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aurens*, *Mycobacterium phlei*
Calotropis procera (Ethanolic extract)	*Enterobacter cloacae*, *Fusarium moniliforme*
Adhatoda vasica	*Pseudomonas fluorescens*
Pericarpium granati, *Rhizoma sanguisorbae*, *Fructus schizandrae*, *Rheum officinale*	*Vibrio alginolyticus*
Acorus calamus, *Indigofera aspalathoides*	*Aeromonas hydrophila*
Ulva fasciata	*Vibrio parahaemolyticus*
Allium sativum, *Magnifera indica* kernel, *Azadirachta indica*	*Aeromonas hydrophila*
Neem extract	Vaccinia virus, Chikungunya, Measles and Coxsackie viruses

(continued)

Table 1
(continued)

Name of plant	Name of microorganism
Maclura cochinchinensis, *Centella asiatica*, *Mangifera indica*, *Cynometra cloiselli*, *C. madagascariensis*, *Ravensara retusa*, *Evonymopsis longipes*, *Terminalia monoceros*, *Acorus calamus* extracts	Herpes Simplex virus
Syzygium kurzi, *Syzygium* megacarapum (extract)	Encephalitis causing virus
Scilla hyacianthiana, *Dillemia pomifera*, *Smilax perfoliadour*, *Rosa osmastonii* (extract)	Semilike Forest virus (SFV), Ranikhet diseases virus (RDV)
Cyanodon dactylon, *Aegle marmelos*, *Tinosporacordifolia*, *Picrorhiza kurooa*, *Eclipta alba*	White spot syndrome virus (WSSV) challenged *Penaeus monodon*
Ocimum sanctum (extract)	*Anti-viral property*
Geranium sanguineum(polyphenolic extracts)	*Various strains of human*, *avian* and *Equine Enfluenza virus*
Hyptianthera stricta (Ethanolic extract)	*Encephalomyocarditis* and *Japanese Encephalitis virus*
Anti-WSSV drugs (MP07X) derived from the marine plant and (TP22C) derived from terrestrial plant	WSSV infected *Litopenaeus vannamei*
Anti-WSSV drug (TP22C) derived from terrestrial plants	WSSV infected *Litopenaeus vannamei*
Fructus prunusis	Black gill disease in farm shrimp *Platanus orientalis* caused by *Fusarium oxysporum*
Helenium quadridentatum (extract)	Saprolegniasis in fish
Neem leaf (extract), oil and seed	*Trichophyton*, *Epidermophyton*, *Micrsporum*, *Trichosporon*, *Geotircum*, *Candida Deuteromyceteous*
Turmeric oil	*Dermatophytic fungi*, *Trichophyton rubrum*
Chenopodium ambrosioides	*Trychophyton mentagrophytes*, *Microsporum audouinii*
Garlic clove extract	*Fusarium solani*
Bauhinia variegate leaf extract	*Aspergillus fumigatus*, *Aspergillus niger*
Nelumbo nucifera	Potent anti-fungal and anti-yeast activity
Eucalyptus citriodora, *Eucalyptus dalrympleana*, *Eucalyptus laveopinea* (essential oil)	Dermatophytes
Eucalyptus citriodora	*Pyricularia grisea*

Adopted from Raman [16]

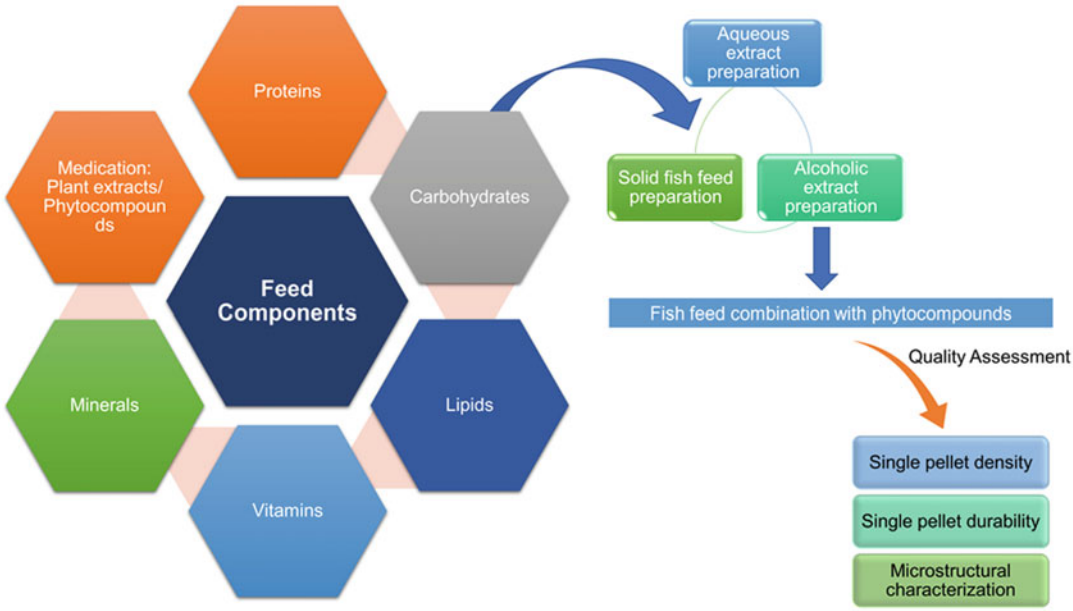

Fig. 1 Overview of feed components, preparation, and characterization

- Fine extracts with fibrous particulates are filtered with fine mesh cloth followed by filtration using Whatman 541 filter paper.
- The crude extracts are collected and stored at 10 °C.

Fish Feed Formulation with Phytocompounds or Plant Extracts [13]

- Various methods for integrating medication differ depending on the type of feed. The medicinal plants/extracts and phytocompounds with antimicrobial activity in aquaculture are enlisted with their target infectious organisms in Tables 2 and 3.
- Dry pellets can be diluted in water or oil before being sprinkled over food [13].
- To make moist pellets, the medication should be thoroughly mixed with the other ingredients in the diet before extrusion.
- Both situations require that medication be evenly distributed and included in the final meal.
- To limit the possibility of medicine leaching, pellets could be coated or dried.
- The medicated feed should be fed to the net pen in a timely and even manner.
- Only active fish will devour the food if it is slowly distributed; sick and inactive fish will starve to death.
- Feed should be supplied in a suitable ratio and at two or more feedings each day to ensure continuous acceptance.

Table 2
Antimicrobial molecules studied for their antiparasitic activity for aquaculture

Phytoconstituent	Source (botanical name)	Parasite	Host (common name)	References
Allicin	*Allium sativum*	*Neobenedenia* sp.	*Lates calcarifer* (*Barramundi*)	[17]
Bruceine A and Bruceine D	*Brucea javanica*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[18]
Chelerythrine	*Chelidonium majus*	*Ichthyophthirius multifiliis*	*Ctenopharyngodon Idella* (*Grass carp*)	[19]
Chelerythrine and chloroxylonine	*Toddalia asiatica*	*Ichthyophthirius multifiliis*	*Carassius auratus* (*Goldfish*)	[20]
Dihydrosanguinarine and dihydrochelerythrine	*Macleaya microcarpa*	*Ichthyophthirius multifiliis*	*Squaliobarbus curriculus* (*Barbel chub*)	[21]
Dioscin and polyphyllin D	*Paris polyphylla*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[19]
Ginkgolic acids (C13: 0 and C15:1)	*Ginkgo biloba*	*Pseudodactylogyrus*	*Anguilla* (*Common eel*)	[22]
Gracillin and Trillin	*Dioscorea zingiberensis*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[23]
Osthol and isopimpinellin	*Fructus cnidii*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[24]
Osthole	*Radix angelicae pubescentis*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[25]
Pentagalloylglucose	*Galla chinensis*	*Ichthyophthirius multifiliis*	*Ictalurus punctatus* (*Channel catfish*)	[26]
Piperine	*Piper longum*	*Argulus* spp.	*Carassius auratus* (*Goldfish*)	[27]
Sanguinarine	*Macleaya microcarpa* *Macleaya cordata*	*Dactylogyrus intermedius* *Ichthyophthirius multifiliis*	*Carassius auratus* (*Goldfish*) *Ctenopharyngodon idella* (*Grass carp*)	[28]

The powdered ingredients are dried at 70–100 °C in an oven for 24 h. The ingredients are individually weighed and mixed thoroughly using a hand mixer. The mixed composition is subjected to densification into pellets. Single pellets are produced using a pelletizing apparatus The pellets are then stored in a sealed plastic container at 4 °C until used [14]. Meanwhile, the application mode of the phytocompounds is based on the nature of the cultivation employed as follows:

Table 3
Antimicrobial plant extracts studied for their antiparasitic activity for aquaculture

Type of extract	Source (botanical name)	Parasite	Host (common name)	References
Aqueous extract	*Cinnamomum cassia*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[29]
Aqueous extract	*Capsicum frutescens*	*Ichthyophthirius multifiliis*	*Carassius auratus* (*Goldfish*)	[30]
Aqueous extract	*Camellia sinensis*	*Trichodina* sp.	*Oreochromis niloticus* (*Nile tilapia*)	[31]
Crude extract	*Allium sativum and Terminalia catappa*	*Trichodina* sp.	*Oreochromis niloticus* (*Nile tilapia*)	[32]
Crushed garlic cloves	*Allium sativum*	*Trichodina* sp. *Gyrodactilus* sp.	*Oreochromis niloticus* (*Nile tilapia*)	[33]
Essential oil	*Melaleuca alternifolia*	*Uronema* sp. *Ichthyophthirius multifiliis*	*Pampus argenteus* (*Silver pomfret*) *Piaractus mesopotamicus* (*Pacu*)	[34]
Essential oil	*Origanum minutiflorum*	*Myxobolus* sp.	*Diplodus puntazzo* (*Sheephead bream*)	[35]
Ethanol extract	*Artemisia annua*	*Monogenea*	*Heterobranchus longifilis* (*Vundu*)	[36]
Freeze-dried	*Allium sativum*	*Gyrodactylus turnbulli*	*Poecilia reticulate* (*Guppy*)	[13]
Methanol extract	*Radix Bupleuri chinensis*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[36]
Methanol extract	*Magnolia officinalis and Sophora alopecuroides*	*Ichthyophthirius multifiliis*	*Carassius auratus* (*Goldfish*)	[37]
Methanol extract	*Dryopteris crassirhizoma*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[38]
Methanol extract	*Piper guineense*	*Gyrodactylus elegans and Dactylogyrus extensus*	*Carassius auratus* (*Goldfish*)	[29]
Methanol extract aqueous extract	*Semen aesculi*	*Dactylogyrus intermedius*	*Carassius auratus* (*Goldfish*)	[38]
Methanol extract petroleum-ether extract	*Mucuna pruriens Carica papaya*	*Ichthyophthirius multifiliis*	*Carassius auratus* (*Goldfish*)	[39]

Water Immersion

1. Bath immersion method: Based on the requirement of the medicinal value for long exposure. The phytoconstituent must be solubilized and essentially dispersed with the culture media. For such requirements the application of phytoconstituents.
2. Dip method of application is for short intervals of exposure, wherein brief contact with a chemical that must be soluble is introduced into the aquaculture system.
3. Flush method: Provision of the medicinal feed through flush for a certain dosage requirement at a certain duration of time in maintenance of the aquaculture.
4. Encapsulation method: Herein, the constituents are available for a longer stay and controlled release into the medium and considering to be relatively significant in having lesser danger of environmental hazards. But requires the appetizing ingredients to be added into the feed.

Medicated Feed

Direct dosage may be provided as an injection, oral route, or topical application. This method of application requires expertise in handling the living fish.

(a) Injection is feasible only with large fish with moderate use of the constituent as per the requirements per body weight.

(b) Oral mode of application is cumbersome and requires appetizing ingredients.

(c) Topical application for region-specific action but requires formulation preparation.

3.2 Aqua Feed Characterization

1. A variety of factors influence aqua feed quality, viz., handling, processing, and storage of feedstuffs, as well as a variety of market-related factors, which can all have an impact on feedstuff quality and safety. Before raw materials can be purchased, quality, traceability, environmental sustainability, and safety standards must be met.
2. Factory-produced feed has always been of high quality, but the quality may have deteriorated by the time it reaches a grower's pond, i.e., commercial fish feed purchased stored outside in bins on large farms. The nutritional value degrades over time, diminishing palatability and aesthetics while also falling below minimum criteria.
3. Long-term storage, among other things, can result in mold growth, vitamin deterioration, and lipid rancidity. Unnecessary handling destroys the feed bags and creates dust, which the fish do not ingest and is thrown away.

Table 4
The feed components with their units

Feed component	Unit
Protein	%
Fat	%
Fibre	%
Ash	%
Macro minerals (calcium, phosphorus, sodium, etc.) Phosphorus is usually expressed on an available basis	%
Trace minerals (zinc, manganese, iron, copper, selenium, etc.,)	mg/kg
Amino acids (arginine, histidine, lysine, methionine, etc.,) The amino acids are usually expressed on a digestible basis.	%
Fatty acids and other lipid components (linoleic acid, linolenic acid, EPA, DHA, cholesterol, phospholipids, etc.,)	%
Starch and non-starch polysaccharides	%
Energy (usually expressed as digestible energy for aquafeeds)	kcal/kg or MJ/kg
Vitamins (vitamin A, thiamine, riboflavin, etc.)	IU/kg, mg/kg, or μg/kg
Phytocompound(s) or plant extract(s)	mg/kg

4. Storage must be pest-free to prevent infection. Simple storage is the most crucial part of conserving the items in good shape.

Feed Composition

Feed composition is analyzed by standard methods of proximate analysis followed as per the guidelines from the Association of Official Analytical Chemists (AOAC). The feed composition and their concentration units are provided in Table 4.

Single Pellet Density

The pellets are measured for their individual weight using weighing balance. The height and diameter are measured with a caliper. The compact density for each formulation is calculated using the formula given below and expressed as kg^{-3} [14]. This enables comparison of medicinal feed preparation along with traditionally used commercial feed.

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}}$$

Single Pellet Durability

Durability of individual pellet is measured using the following formula [14]. The durability changes as per the protocol followed for a particular application procedure employed.

$$\text{Durability } (\%) = \frac{W_f}{W_i} \times 100$$

W_f = Final weight; W_i = Initial weight.

Microstructural Characterization

The scanning electron microscope is used to observe the morphology of the pellet powder ingredients [14]. This can further be used for the assessment of action of the medicinal feed on the required antimicrobial activity [15].

4 Conclusion

In conclusion, the medicinal plants have been used in the treatment of parasitic, bacterial, viral, and fungal infections in fish and shrimps. Feed formulation with effective doses of antimicrobial plant extracts or phytocompounds provide the best treatment strategies for aquaculture of fish and shrimps. Since the medicinal plants are well-known for their cultural acceptability and lesser adverse effects on human health, integrating effective antimicrobial components during feed formulation is essential.

References

1. Naylor RL, Hardy RW, Buschmann AH, Bush SR, Cao L, Klinger DH et al (2021) A 20-year retrospective review of global aquaculture. Nature 591:551–563
2. Boyd CE, D'Abramo LR, Glencross BD, Huyben DC, Juarez LM, Lockwood GS et al (2020) Achieving sustainable aquaculture: historical and current perspectives and future needs and challenges. J World Aquacult Soc 51:578–633
3. Hua K, Cobcroft JM, Cole A, Condon K, Jerry DR, Mangott A et al (2019) The future of aquatic protein: implications for protein sources in aquaculture diets. One Earth 1: 316–329
4. Mzula A, Wambura PN, Mdegela RH, Shirima GM (2021) Present status of aquaculture and the challenge of bacterial diseases in freshwater farmed fish in Tanzania; a call for sustainable strategies. Aquacult Fish 6:247–253
5. Okocha RC, Olatoye IO, Adedeji OB (2018) Food safety impacts of antimicrobial use and their residues in aquaculture. Public Health Rev 39:21
6. Wang C, Li Z, Wang T, Xu X, Zhang X, Li D (2021) Intelligent fish farm—the future of aquaculture. Aquacult Int 29:2681–2711
7. Schar D, Klein EY, Laxminarayan R, Gilbert M, Van Boeckel TP (2020) Global trends in antimicrobial use in aquaculture. Sci Rep 10: 21878
8. Marshall BM, Levy SB (2011) Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev 24:718–733
9. Awad E, Awaad A (2017) Role of medicinal plants on growth performance and immune status in fish. Fish Shellfish Immunol 67:40–54
10. Venmathi Maran BA, Josmeh D, Tan JK, Yong YS, Shah MD (2021) Efficacy of the aqueous extract of Azadirachta indica against the marine

parasitic leech and its phytochemical profiling. Molecules 26:1908

11. Rashidian G, Boldaji JT, Rainis S, Prokić MD, Faggio C (2021) Oregano (Origanum vulgare) extract enhances zebrafish (Danio rerio) growth performance, serum and mucus innate immune responses and resistance against Aeromonas hydrophila challenge. Animals 11:299
12. Pandey G (2013) Feed formulation and feeding technology for fishes. Int Res J Pharm 4: 23–30
13. Schelkle B, Snellgrove D, Cable J (2013) In vitro and in vivo efficacy of garlic compounds against Gyrodactylus turnbulli infecting the guppy (Poecilia reticulata). Vet Parasitol 198: 96–101
14. Zettl S, Cree D, Soleimani M, Tabil L (2019) Mechanical properties of aquaculture feed pellets using plant-based proteins. Yildiz F (ed). Cogent Food Agric 5:1656917
15. Chen Y, Wu J, Cheng H, Dai Y, Wang Y, Yang H et al. Anti-infective effects of a fish-derived antimicrobial peptide against drug-resistant bacteria and its synergistic effects with antibiotic. Front Microbiol [Internet]. 2020 [cited 2022 Feb 9];11. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2020.602412
16. Raman RP (2017) Applicability, feasibility and efficacy of phytotherapy in aquatic animal health management. Am J Plant Sci 8:257–287
17. Valenzuela-Gutiérrez R, Lago-Lestón A, Vargas-Albores F, Cicala F, Martínez-Porchas M (2021) Exploring the garlic (Allium sativum) properties for fish aquaculture. Fish Physiol Biochem 47:1179–1198
18. Nakao R, Mizukami C, Kawamura Y, Subeki, Bawm S, Yamasaki M et al (2009) Evaluation of efficacy of bruceine A, a natural quassinoid compound extracted from a medicinal plant, Brucea javanica, for canine babesiosis. J Vet Med Sci 71:33–41
19. Li X-L, Yao J-Y, Zhou Z-M, Shen J-Y, Ru H-S, Liu X-L (2011) Activity of the chelerythrine, a quaternary benzo[c]phenanthridine alkaloid from Chelidonium majus L. on Dactylogyrus intermedius. Parasitol Res 109:247–252
20. Shan X, Meng Q, Kang Y, Bian Y, Gao Y, Wang W et al (2014) Isolation of active compounds from methanol extracts of Toddalia asiatica against Ichthyophthirius multifiliis in goldfish (Carassius auratus). Vet Parasitol 199:250–254
21. Kang Y-J, Yi Y, Zhang C, Wu S-Q, Shi C-B, Wang G-X (2013) Bioassay-guided isolation and identification of active compounds from Macleaya microcarpa (maxim) Fedde against fish pathogenic bacteria. Aquac Res 44:1221–1228
22. Wang G-X, Jiang D, Zhou Z, Zhao Y-K, Shen Y-H (2009) In vivo assessment of anthelmintic efficacy of ginkgolic acids (C13:0, C15:1) on removal of pseudodactylogyrus in European eel. Aquaculture [Internet]. Amsterdam: Elsevier Science; [cited 2022 Feb 9]. Available from: doi:https://doi.org/10.1016/j.aquaculture.2009.09.012
23. Wang G-X, Jiang D-X, Li J, Han J, Liu Y-T, Liu X-L (2010) Anthelmintic activity of steroidal saponins from Dioscorea zingiberensis C. H. Wright against Dactylogyrus intermedius (Monogenea) in goldfish (Carassius auratus). Parasitol Res 107:1365–1371
24. Wang G, Zhou Z, Cheng C, Yao J, Yang Z (2008) Osthol and isopimpinellin from Fructus cnidii for the control of Dactylogyrus intermedius in Carassius auratus. Vet Parasitol 158: 144–151
25. Liu G-L, Liu L, Hu Y, Wang G-X (2021) Evaluation of the antiparasitic activity of coumarin derivatives against Dactylogyrus intermedius in goldfish (Carassius auratus). Aquaculture 533: 736069
26. Zhang Q, Xu D-H, Klesius PH (2013) Evaluation of an antiparasitic compound extracted from Galla chinensis against fish parasite Ichthyophthirius multifiliis. Vet Parasitol 198: 45–53
27. Kumar A, Raman RP, Kumar K, Pandey PK, Kumar V, Mohanty S et al (2012) Antiparasitic efficacy of piperine against Argulus spp. on Carassius auratus (Linn. 1758): in vitro and in vivo study. Parasitol Res 111:2071–2076
28. Zhang R, Wang X-W, Zhu J-Y, Liu L-L, Liu Y-C, Zhu H (2019) Dietary sanguinarine affected immune response, digestive enzyme activity and intestinal microbiota of Koi carp (cryprinus carpiod). Aquaculture 502:72–79
29. Ji J, Lu C, Kang Y, Wang G-X, Chen P (2012) Screening of 42 medicinal plants for in vivo anthelmintic activity against Dactylogyrus intermedius (Monogenea) in goldfish (Carassius auratus). Parasitol Res 111:97–104
30. Ling F, Wang J-G, Lu C, Wang G-X, Lui Y-H, Gong X-N (2012) Effects of aqueous extract of Capsicum frutescens (Solanaceae) against the fish ectoparasite Ichthyophthirius multifiliis. Parasitol Res 111:841–848
31. Abdel-Tawwab M, Ahmad MH, Seden MEA, Sakr SFM (2010) Use of green tea, Camellia sinensis L., in practical diet for growth and protection of Nile tilapia, Oreochromis niloticus (L.), against Aeromonas hydrophila infection. J World Aquacult Soc. 41:203–213

32. Chitmanat C, Nunsong W, Tongdonmuan K (2005) The use of crude extracts from traditional medicinal plants to eliminate Trichodina sp. in tilapia (Oreochromis niloticus) fingerlings. Songklanakarin. J Sci Technol 27:359–364
33. Abd El-Galil MAA, Aboelhadid SM (2012) Trials for the control of trichodinosis and gyrodactylosis in hatchery reared Oreochromis niloticus fries by using garlic. Vet Parasitol 185:57–63
34. Valladão GMR, Gallani SU, Ikefuti CV, da Cruz C, Levy-Pereira N, Rodrigues MVN et al (2016) Essential oils to control ichthyophthiriasis in pacu, Piaractus mesopotamicus (Holmberg): special emphasis on treatment with Melaleuca alternifolia. J Fish Dis 39: 1143–1152
35. Karagouni E, Athanassopoulou F, Lytra A, Komis C, Dotsika E (2005) Antiparasitic and immunomodulatory effect of innovative treatments against Myxobolus sp. infection in Diplodus puntazzo. Vet Parasitol 134:215–228
36. Ekanem AP, Brisibe EA (2010) Effects of ethanol extract of Artemisia annua L. against monogenean parasites of Heterobranchus longifilis. Parasitol Res 106:1135–1139
37. Yi Y-L, Lu C, Hu X-G, Ling F, Wang G-X (2012) Antiprotozoal activity of medicinal plants against Ichthyophthirius multifiliis in goldfish (Carassius auratus). Parasitol Res 111:1771–1778
38. Jiang B, Chi C, Fu Y, Zhang Q, Wang G (2013) In vivo anthelmintic effect of flavonol rhamnosides from Dryopteris crassirhizoma against Dactylogyrus intermedius in goldfish (Carassius auratus). Parasitol Res 112:4097–4104
39. Ekanem AP, Obiekezie A, Kloas W, Knopf K (2004) Effects of crude extracts of Mucuna pruriens (Fabaceae) and Carica papaya (Caricaceae) against the protozoan fish parasite Ichthyophthirius multifiliis. Parasitol Res 92: 361–366

Part V

Aquatic Actinomycetes and Copepods

Chapter 23

Isolation and Identification of *Actinomycetes*

Haimanti Mondal, John Thomas, and Natarajan Amaresan

Abstract

Actinomycetes are Gram-positive, facultatively anaerobic bacteria since most of them grow best under anaerobic conditions. They consist of mycelium in the filamentous form with branched growth pattern. This species may form endospores and have either coccoid- or rod-shaped forms. Some species may be acidophilic, thermophilic, and alkaliphilic. They are often found in moderate pH levels and mostly prefer moderate temperatures. *Actinomycetes* form symbiotic associations with various plant species by nitrogen fixation. They have wide applications in various fields and are used as a source of antibiotics, pesticides, and so on.

Key words *Actinomycetes*, Filamentous, Mycelium, Endospore, Facultatively anaerobe

1 Introduction

Actinomycetes are generally Gram-positive ubiquitous, spore-forming bacteria with diverse biological activities [1]. They are placed within the phylum *Actinobacteria*, class *Actinomycetia*, subclass *Actinobacteridae*, and order *Actinomycetales* that consists of over 160 genera, 10 suborders, and more than 30 families [2]. They are considered to be one of the vast bacterial phyla consisting of filamentous fungal-like morphological characteristics [3]. They are known to have over 55% high guanine to cytosine (G + C) content taxonomically [1, 4]. The most common genera of *Actinomycetes* include *Streptomyces*, *Actinomyces*, *Micrococcus*, and *Actinoplanes* [5, 6].

Actinomycetes are an immense source of microbial resources that have huge commercial value and vast practical use. They have huge biosynthetic potential to produce secondary metabolites [7]. They are the source of approximately 70% of antibiotics and nonantibiotic bioactive metabolites like enzymes, anti-oxidation reagents, enzyme inhibitors, immunological regulators, etc. [2].

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_23,

[8] reported that *Actinomycetes* are widely distributed in both terrestrial and marine habitats, especially in the soil and oceans. The best marine source of *Actinomycetes* reported is the sediment. The isolation of *Actinomycetes* has also been reported from water, rocks, sand, deep sediment, mangrove sediment, marine plants, and seafoods [2]. They are used in the production of secondary metabolites and their products are widely used for different treatments [9].

2 Materials

2.1 Isolation of Actinomycetes [10]

- Sediment sample.
- Autoclave bags.
- Test tubes.
- Petri plates.
- Actinomycetes isolation agar (AIA) medium (sodium caseinate, 2 g; L-asparagine, 0.1 g; sodium propionate, 4 g; dipotassium phosphate, 0.5 g; magnesium sulfate, 0.1 g; ferrous sulfate, 0.001 g; agar, 15 g; final pH, 8.1 ± 0.2; distilled water, 1 L).
- ISP2 broth.
- Sodium chloride (NaCl).
- Nalidixic acid.
- Mycostatin.

3 *Actinomycetes* Identification [11–13]

3.1 Staining Methods

- Gram staining kit and capsule staining (refer to standard microbiology laboratory manual).

3.2 Biochemical Tests

- KB003 Hi 25 Biochemical kit.
- KB009A/KB009B/KB009C Hi Carbo™ kit.

3.3 Molecular Identification

- PCR reaction mix as per the standard protocols.
- Universal primers 27F and 1492R.
- Conserved universal primer 800R.
- Thermocycler.
- Transilluminator.

4 Methods

4.1 Pretreatment of Marine Sediment Samples

- Pour the sediment sample in a glass petri plate.
- Keep the sample overnight at 70 °C in hot air oven for drying.
- Under aseptic condition, crush and grind the samples aseptically using mortar and pestle.

4.2 Isolation of Actinomycetes *from Marine Sediments/Soil*

- Weigh 1 g of sediment/soil sample.
- Disperse the sample into 100 mL conical flask containing 0.9% saline with distilled water.
- Keep at room temperature for 10 min using an orbital shaker.
- Prepare serial dilution.
- Transfer 0.1 mL aliquot of the last four dilutions on AIA medium supplemented with nalidixic acid (50 μg/mL) and mycostatin (100 μg/mL).
- Incubate the petri plates at 30 °C for 7–14 days.
- Select the pure colonies and subculture them from the master plate.

4.3 Identification of Actinomycetes

4.3.1 Staining Methods and Biochemical Tests

- Perform the staining techniques and biochemical tests as per standard procedures.

4.3.2 Scanning Electron Microscopy (SEM) Analysis

- Inoculate the isolate in the broth medium.
- Incubate the culture medium in rotary shaker at 30 °C for 7 days until growth is visible.
- Centrifuge the broth culture at 7000 rpm for 10 min and discard the supernatant.
- Add 1 mL autoclaved phosphate-buffered saline (PBS) in cell-free supernatant and mix well.
- Transfer 10–20 μL of the culture on a grease-free clean slide.
- Dispense 20 μL of 0.25% of glutaraldehyde solution into the slide.
- Oven-dry the slide for 48 h at 40 °C.

4.3.3 Molecular Identification [14, 15]

- Extract the genomic DNA from *Actinomycetes* using lysis method by boiling suspensions of bacterial culture cells as per the standard protocols.
- Perform 16S rRNA gene amplification with a reaction mix of 50 μL containing 1 μL of genomic DNA, 0.2 μM of each primer, and 25 μL Master Mix using universal primers.

- Amplification reaction starts with an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 1.5 min with final extension at 72 °C for 10 min.
- Purify the PCR products and sequence it using conserved universal primer 800R.
- Edit the retrieved sequences manually and perform BLAST nucleotide analyses with NCBI to identify the *Actinomycetes* up to the genus level.

References

1. Daquioag JEL, Penuliar GM (2021) Isolation of *Actinomycetes* with cellulolytic and antimicrobial activities from soils collected from an urban green space in the Philippines. Int J Microbiol 2021:6699430
2. Dilip VC, Mulaje SS, Mohalkar RY (2013) A review on *Actinomycetes* and their biotechnological application. Int J Pharm Sci Res 4(5): 1730–1742
3. Jose PA, Jha B (2016) New dimensions of research on *Actinomycetes*: quest for next generation antibiotics. Front Microbiol 7:1295
4. Abdul MKN, Chowdhury AJK, Zainuddin Z, Abidin ZAZ (2014) Selective isolation of *Actinomycetes* from mangrove forest of Pahang, Malaysia. In: Proceedings of the international conference on agriculture, biology and environmental sciences (ICABES'14), Bali, Indonesia, pp 8–9
5. Agadagba SK (2014) Isolation of *Actinomycetes* from soil. J Microbiol Res 4(3):136–140
6. Adegboye MF, Babalola OO (2012) Taxonomy and ecology of antibiotic producing actinomycetes. Afr J Agric Res 7(15):2255–2261
7. Niu G (2018) Genomics-driven natural product discovery in *Actinomycetes*. Trends Biotechnol 36:238–241
8. Chandramohan D (1991) Coastal microbial processe. In: Natarajan R, Dwivedi SN, Ramachandran S (eds) Coastal zone management (in Tamilnadu state, India). Ocean Data Centre, Anna University, Madras, p 93
9. Shrestha B, Nath DK, Maharjan A, Poudel A, Pradhan RN et al (2021) Isolation and characterization of potential antibiotic-producing *Actinomycetes* from water and soil sediments of different regions of Nepal. Int J Microbiol 5586165:1–9
10. Singh V, Haque S, Singh H, Verma J, Vibha K et al (2016) Isolation, screening, and identification of novel isolates of *Actinomycetes* from India for antimicrobial applications. Front Microbiol 7:1921
11. Cheesbrough M (2010) Adaptive or specific immune response, district laboratory in tropical countries, 2nd edn. Cambridge University Press, Cape Town, p 11
12. Rajkumar T, Manimaran M, Taju G, Vimal S, Majeed AS et al. (2018) Antiviral viral compound from *Streptomyces ghanaensis* like strain against white spot syndrome virus (WSSV) of shrimp. bioRxiv https://doi.org/10.1101/340265
13. Undabarrena A, Beltrametti F, Claverías FP, González M, Moore ERB et al (2016) Exploring the diversity and antimicrobial potential of marine Actinobacteria from the Comau Fjord in northern Patagonia. Chile Front Microbiol 7:1135
14. Claverías FP, Undabarrena A, González M, Seeger M, Cámara B (2015) Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaíso bay. Chile Front Microbiol 6:737
15. Moore E, Arnscheidt A, Krüger A, Strömpl C, Mau M (2004) Simplified protocols for the preparation of genomic DNA from bacterial cultures. In: Molecular microbial ecology manual, vol. 1. Kluwer Academic Publishers, Dordrecht, pp 3–18

Chapter 24

Assay of Hemolytic Activity

Haimanti Mondal, John Thomas, and Natarajan Amaresan

Abstract

Hemolysis refers to the breakdown of erythrocytes/red blood cells (RBCs). This results in the destruction of erythrocytes, releasing hemoglobin from within RBCs into the blood plasma. In vitro hemolysis is the result of pre-analytical causes associated with parameters including extreme temperature, sample collection and handling, jarring transportation techniques, delayed processing, and later prolonged storage. Hemolytic assays are performed on bacteria including *Actinomycetes* after the extraction of secondary metabolites with the most potent solvent. Later, they are screened for hemolytic activity against bacterial and fungal pathogens in aquaculture.

Key words Hemolysis, In vitro *hemolysis*, *Red blood cells*, *Hemolytic assays*

1 Introduction

One of the major virulence factors, hemolysins, is considered as the compounds produced by a wide variety of bacterial species including *Actinomycetes*. They are responsible for cell lysis, destruction of adjacent cells and tissues, and membrane damage in order to supply nutrients like iron for toxin producers [1].

Hemolytic assays of *Actinomycetes* can be performed on the blood of marine animals [2–5]. Several studies have been reported on *Actinomycetes* where the secondary metabolites are extracted and screened for hemolytic activity against particular bacterial and fungal pathogens. Suthindhiran and Kannabiran [6] reported the in vitro hemolytic activity of secondary metabolites extracted from several species of *Actinomycetes* including *Streptomyces*, *Micromonospora*, *Actinopolyspora*, and *Saccharopolyspora* on mouse RBCs.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_24,

2 Materials

- Petri plates.
- Sheep blood agar (SBA) medium (casein enzymic hydrolysate, 14 g; peptic digest of animal tissue, 4.5 g; yeast extract, 4.5 g; sodium chloride, 5 g; sheep blood, 0.5 g; agar, 12.5 g; final pH, 7.3 ± 0.2; distilled water, 1 L).
- Sheep/mouse erythrocytes.
- ISP2 broth.
- 96-well plates.
- Calcium chloride ($CaCl_2$).
- Sodium chloride (NaCl).
- Triton X-100.
- 10× phosphate-buffered saline (PBS).

3 Methods

3.1 Hemolytic Assay [7]

- Inoculate the actinomycetes' isolates on 5% sheep blood agar medium.
- Incubate at room temperature at 28–30 °C for 2–3 days.
- Record the zone of inhibition (in mm) around the actinomycetes' colonies which indicates hemolytic activity.

3.2 In Vitro Hemolytic Assay [6, 8–10]

- Wash the erythrocyte fraction thrice with saline and resuspend in 10 mL PBS.
- Perform the hemolytic activity of the bioactive compound by hemolytic assay in 96-well plates.
- Pour 100 μL of 0.85% NaCl solution containing 10 MM $CaCl_2$ in each well.
- Consider the first well as negative control that contains only water.
- Add 100 μL of various concentrations (5–500 μg/mL) of bioactive compounds in the second well.
- Adjust the osmolarity of the extract with 10× PBS for preventing osmotic lysis of RBCs.
- Take the last well as positive control containing 20 μL of 0.1% Triton X-100 in 0.85% saline.
- Add 100 μL of a 2% suspension of human/mouse erythrocytes in 0.85% saline containing 10 MM $CaCl_2$ in each well.

- Incubate at room temperature for 30 min to 2 h.
- Centrifuge and remove the supernatant.
- Record the absorbance of the liberated hemoglobin at 540 nm.

References

1. Mogrovejo DC, Perini L, Gostinčar C, Sepčić K, Turk M et al (2020) Prevalence of antimicrobial resistance and hemolytic phenotypes in culturable arctic bacteria. Front Microbiol 11:570
2. Norouzi H, Danesh A, Mohseni M, Khorasgani MR (2018) Marine *Actinomycetes* with probiotic potential and bioactivity against multidrug-resistant bacteria. Int J Mol Cell Med 7:44–52
3. Jagannathan SV, Manemann EM, Rowe SE et al (2021) Marine *Actinomycetes*, new sources of biotechnological products. Mar Drugs 19(7):365
4. Bernal MG, Campa-Córdova ÁI, Saucedo PE, González MC, Marrero RM et al (2015) Isolation and in vitro selection of actinomycetes strains as potential probiotics for aquaculture. Vet World 8:170–176
5. Das S, Ward LR, Burke C (2010) Screening of marine *Streptomyces* spp. for potential use as probiotics in aquaculture. Aquaculture 305: 32–41
6. Suthindhiran K, Kannabiran K (2010) Diversity and exploration of bioactive marine *Actinomycetes* in the Bay of Bengal of the Puducherry coast of India Indian. J Microbiol 50(1): 76–82
7. Fatmawati U, Meryandini A, Nawangsih AS, Wahyudi AT (2010) Screening and characterization of *Actinomycetes* isolated from soybean rhizosphere for promoting plant growth. Biodivers J Biol Divers 20(10):2970–2977
8. Costa-Lotufo LV, Cunha GMA, Farias PAM, Viana GSB, Cunha KMA, Pessoa C, Moraes MO, Silveira ER, Gramosa NV, Rao VSN (2002) The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oloeo-resin. Toxicon 40:1231–1234
9. Saurav K, Kannabiran K (2012) Cytotoxicity and antioxidant activity of 5-(2,4-dimethylbenzyl)pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp. Saudi J Biol Sci 19: 81–86
10. Malagoli D (2007) A full-length protocol to test hemolytic activity of palytoxin on human erythrocytes. Invert Surviv J 4:92–94

Chapter 25

Cytotoxicity Assay

Haimanti Mondal, John Thomas, and Natarajan Amaresan

Abstract

Cytotoxicity test is defined as the screening test and biological evaluation of tissue cells in vitro to detect the growth, the reproduction, and also the morphological effects of the cell by use of medical devices. It is performed in vitro for determining whether that particular medical device can cause death of a cell either by direct contact or leaching of several toxic substances. Cytotoxicity assays measure the loss of some cellular and intercellular functions or structures involving lethal cytotoxicity. They also indicate whether they cause both cell and tissue injury. These assays are conducted on several bacteria including *Actinomycetes* as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay against several cell lines to observe their cytotoxicity level.

Key words Cytotoxicity assay, *Actinomycetes*, Lethal, Toxicity

1 Introduction

Analysis of the biological activity of the secondary metabolites reveals the efficacy of the bactericidal agents against several pathogens and also their in vitro cytotoxic activity against a cancerous or tumor cell line in aquaculture [1]. They also focus on the elucidation of the mode of action resulting in the death of a cell highlighting selective cytotoxic activity [2].

Several studies have been reported on the cytotoxic effect of the *Actinomycetes* extract against particular human cell lines [2]. Gozari et al. [3] reported a study where they screened and identified five most potent *Actinomycetes* strains. They later showed that these *Actinomycetes* strains produced antibacterial secondary metabolites with cytotoxic agents against various microbial pathogens and human tumor cell lines (HTCLs). They also observed that the most potent antibacterial extracts of *Actinomycetes* exhibited high dose-dependent cytotoxicity against HTCLs, with either low or no significant toxicity toward normal cells.

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_25,

2 Materials

- 5-(2,4-dimethylbenzyl)pyrrolidine-2-one (DMBPO).
- Roswell Park Memorial Institute 1640 (RPMI 1640) medium/ Dulbecco's Modified Eagle Medium (DMEM).
- HEP2 (laryngeal carcinoma cells), Vero (green monkey kidney), Hep G2 (hepatocellular carcinoma) cell lines.
- Streptomycin.
- Penicillin.
- 10% fetal bovine serum (FBS).
- Cell Quanti-MTT assay kit.
- 96-well tissue culture plate.
- Multi well plate reader.
- Dimethylsulfoxide (DMSO).

3 Methods

3.1 Assay of Cytotoxicity [3, 4]

- Perform the cytotoxicity of DMBPO (0–25 μg/mL) on different cell lines like HEP2 (laryngeal carcinoma cells), Vero (green monkey kidney), and Hep G2 (hepatocellular carcinoma) by MTT cell proliferation assay.
- Culture the cells routinely in 75 cm^2 culture flasks.
- Maintain it in RPMI 1640/DMEM medium supplemented with streptomycin (100 mg/L), penicillin (100 IU/mL), and 10% FBS (v/v) in 5% carbon dioxide at 37 °C.
- Quantify the cell lines (1×10^5 cells/well) according to the user manual.
- Culture the cells (80 mL/well) in a 96-well clear bottom tissue culture plate.
- Incubate till they get confluence.
- Add the test compounds with cells and incubate at different time periods.
- Add 15 mL/80 μL cell culture of Cell Quanti-MTT™ reagent/ well.
- Incubate at 37 °C for 4 h.
- Pour 100 mL of solubilization solution and keep in orbital shaker at room temperature for 1 h.
- Measure the optical density (OD) for each well at 570 nm on a multi well plate reader.
- Perform the test in triplicates.

- Consider the wells treated with 0.1% DMSO or only culture medium as control.
- Determine the average of controls (blank) and subtract from absorbance values.
- Plot the graph with cell viability against time period with different concentrations of the compound.

References

1. Salem SS, El-Beley EF, Niedbala G, Alnoman MM, Hassan et al (2020) Bactericidal and in-vitro cytotoxic efficacy of silver nanoparticles (Ag-NPs) fabricated by endophytic *Actinomycetes* and their use as coating for the textile fabrics. Nanomaterials (Basel) 10(10):2082
2. Ser H-L, Mutalib N-S, Yin W-F, Chan K-G, Goh B-H et al (2015) Evaluation of antioxidative and cytotoxic activities of *Streptomyces pluripotens* MUSC 137 isolated from mangrove soil in Malaysia. Front Microbiol 6:1398
3. Gozari M, Zaheri A, Jahromi ST, Gozari M, Karimzadeh R (2019) Screening and characterization of marine *Actinomycetes* from the northern Oman Sea sediments for cytotoxic and antimicrobial activity. Int Microbiol. https://doi.org/10.1007/s10123-019-00083-3
4. Saurav K, Kannabiran K (2012) Cytotoxicity and antioxidant activity of 5-(2,4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp. Saudi J Biol Sci 19:81–86

Chapter 26

Antibacterial Activity and Extraction of Bioactive Compound from Actinomycetes

Haimanti Mondal, John Thomas, and Natarajan Amaresan

Abstract

Currently, the pathogenic organisms are showing greater acquired resistance against almost all frequently used antibacterial agents. There is a dire need of novel bioactive compounds to fight against such infectious pathogens. To detect and isolate newer bioactive compounds from *Actinomycetes*, many unknown and unexplored areas are explored, and screening of antibacterial agents performed. Till date, several thousands of bioactive compounds have been isolated from *Actinomycetes* and characterized. Many of them have been developed into drugs for the treatment of diseases in aquaculture. Thus, *Actinomycetes* are regarded as the most potent source in the production of antibiotics, secondary metabolites, and other novel bioactive compounds.

Key words Pathogen, Antibacterial, Bioactive compounds, Secondary metabolites, Treatment

1 Introduction

Actinomycetes are well-known for the production of a vast number of bioactive compounds [1]. Almost two-thirds of the antimicrobial compounds that are currently used are produced by several species of *Actinomycetes* [2]. Bioactive molecules are also known as secondary metabolites. They are not important for reproduction and growth but may presumably activate the defense system of the producer microbe to help them compete in the external environment [3]. *Actinomycetes* are considered as one of the most potential sources of novel bioactive metabolites. Almost 70% of the known drugs, of which 60% and 75% are used in agriculture and medicine, are isolated from actinomycetes. They are the prime source of secondary metabolites and in addition have anticellular activities [3].

Actinomycetes have outstanding capacity to deliver the most unique, assorted, and remarkable antibacterial effectiveness with low toxicity [4, 5]. Cho et al. [6] reported several secondary

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_26,

metabolites produced by *Streptomyces* sp. that have been used successfully as antibiotics in treating drug-resistant infections in humans and animals. Several studies reported that secondary metabolites produced by *Actinomycetes* showed very high antibacterial activity [7].

2 Materials

- Petri plates.
- Actinomycetes isolation agar (AIA) medium (sodium caseinate, 2 g; L-asparagine, 0.1 g; sodium propionate, 4 g; dipotassium phosphate, 0.5 g; magnesium sulfate, 0.1 g; ferrous sulfate, 0.001 g; agar, 15 g; final pH, 8.1 ± 0.2; distilled water, 1 L).
- Mueller Hinton agar (MHA) medium (meat infusion from equivalent to beef, 300 g; casein acid hydrolysate, 17.5 g; starch, 1.5 g; agar, 17 g; final pH, 7.3 ± 0.1; distilled water, 1 L).
- ISP2 broth.
- Sodium chloride (NaCl).
- Cork borer.
- Whatman No. 1 filter paper.
- Separating funnel.

3 Methods

3.1 In Vitro Antibacterial Activity [8–10]

- Perform the preliminary screening using cross-streak method to screen the isolates against the pathogens.
- Supplement the agar plates with 2% NaCl.

3.1.1 Primary Screening

- Inoculate the isolate by a single streak in the center of the plate.
- Incubate the petri plates at 30 °C for 7 days.
- Streak the sub-cultured test pathogens perpendicularly at 90° to the *Actinomycetes* isolate.
- Incubate the petri plates at 37 °C for 24 h.
- Record the zone of inhibition (in mm) around the colonies.

3.1.2 Secondary Screening

- Select the *Actinomycetes* isolates showing potential antibacterial activities from the primary screening.
- Inoculate the isolates into ISP2 broth.
- Keep the broth culture in a rotary shaker incubator at 120 rpm, 30 °C for 7 days.

- Centrifuge the culture at 4 °C, 10,000 rpm for 20 min.
- Collect the supernatant.

3.2 Agar Well Diffusion Method

- Spread the lawn cultures of the pathogens on the solidified agar plates using sterile cotton swabs.
- Make wells using a sterile cork borer on the agar plates.
- Add the supernatant (crude extract) to the agar wells.
- Wait until the extracts are completely diffused onto the agar plates.
- Incubate the petri plates at 37 °C for 24 h.
- Conduct the experiment three times and record the mean of zone of inhibition (in mm).

3.3 Extraction of the Bioactive Compound

- Prepare the inoculum by inoculating the selected isolate into ISP2 broth.
- Incubate the culture broth in rotary shaker incubator at 30 °C for 7 days.
- Filter the broth through Whatman No. 1 filter paper.
- Centrifuge the filtrate at 4 °C and 10,000 rpm for 20 min.
- Collect the cell-free supernatant and extract it three times with an equal volume of ethyl acetate in 1:1 ratio using separating funnel.
- Collect the upper layer in a conical flask.
- Keep it in rotary shaker incubator for 24 h.
- Allow to settle for 20 min after taking out from shaker.
- Collect the upper layer.
- Concentrate the solvent layer using rotary evaporator.

References

1. Undabarrena A, Beltrametti F, Claverías FP, González M, Moore ERB et al (2016) Exploring the diversity and antimicrobial potential of marine actinobacteria from the Comau Fjord in Northern Patagonia, Chile. Front Microbiol 7: 1135
2. Bentley SD, Chater KF, Cerdeño-Tárraga A-M, Challis GL, Thomson NR et al (2002) Complete genome sequence of the model *Actinomycete Streptomyces coelicolor* A3(2). Nature 417(6885):141–147
3. Kumar PS, Raj JPP, Duraipandiyan V, Ignacimuthu S (2012) Antibacterial activity of some *Actinomycetes* from Tamil Nadu, India. Asian Pac J Trop Biomed 2(12):936–943
4. Tanvir R, Sajid I, Hasnain S, Kulik A, Grond S (2016) Rare *Actinomycetes Nocardia caishijiensis* and *Pseudonocardia carboxydivorans* as endophytes, their bioactivity and metabolites evaluation. Microbiol Res 185:22–35
5. Kumari N, Menghani E, Mithal R (2019) Bioactive compounds characterization and antibacterial potentials of *Actinomycetes* isolated from rhizospheric soil. J Sci Ind Res 78:793–798

6. Cho SS, Choi YH, Simkhada JR, Mander P, Park DJ et al (2012) A newly isolated *Streptomyces* sp. CS392 producing three antimicrobial compounds. Bioprocess Biosyst Eng 35:247–254
7. Ravi L, Krishnan K (2016) Bioactivity-guided extraction and identification of antibacterial compound from marine *Actinomycetes* strains isolated from costal soil samples of Rameswaram and Dhanushkodi, Tamil Nadu, India. Asian J Pharm 10(4):S504–S509
8. Thirumurugan D, Vijayakumar R (2013) A potent fish pathogenic bacterial killer *Streptomyces* sp. isolated from the soils of east coast region, South India. J Coast Life Med 1(3): 175–180
9. Dholakiya RN, Kumar R, Mishra A, Mody KH, Jha B (2017) Antibacterial and antioxidant activities of novel Actinobacteria strain isolated from Gulf of Khambat, Gujarat. Front Microbiol 8:2420
10. Rajkumar T, Manimaran M, Taju G, Vimal S, Majeed AS et al (2018) Antiviral viral compound from *Streptomyces ghanaensis* like strain against white spot syndrome virus (WSSV) of shrimp. bioRxiv. https://doi.org/10.1101/340265

Chapter 27

Isolation and Identification of Harpacticoid Copepod

M. F. Yasmeen and A. Saboor

Abstract

Harpacticoid copepods are crustaceans that include benthic as well as planktonic forms. They are nutrient-rich live food organisms used in finfish and shellfish culture. They feed on diverse food sources such as microalgae, fungi, protozoans, bacteria, and detritus. These copepods can able to sustain themselves even during the low availability of their food sources. Many harpacticoid species have been recognized to possess characteristic features that make them more suitable as a live feed. This chapter aims to exemplify a simple protocol to isolate and identify the harpacticoid copepods.

Key words Copepod, Harpacticoid, Live feed, Aquaculture

1 Introduction

In an aquatic ecosystem, copepods constitute the major percentage of consumer biomass. They are known as the essential key linkers due to their ability to transfer energy from primary producers to secondary consumers precisely to fish larvae and juveniles [1]. Studies on the larval stages of the majority of fishes revealed that they rely on copepods for their primary food source [2].

Copepods are a natural synthesizer of essential fatty acids like polyunsaturated fatty acid (PUFA), viz., eicosapentaenoic acid (20:5 ω-3) and docosahexaenoic acid (22:6 ω-3) [3]. Many copepod species play a crucial role in aquaculture as they possess high reproducing capacity, larger brood size, longer reproducing duration, more female population, shorter generation time, shorter turnover time, faster growth, and high survival rate [4]. All these features are found to be persisting in many harpacticoid copepods [5]. They can feed on readily available food in nature including the detrital organic matter and microorganisms such as bacteria, algae, and protozoans. Bacteria are also one of the essential food components for the harpacticoid copepods [6].

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7_27,

Many aquafarmers depend on imported feeds for aquaculture which are not only expensive but may also have a risk of introducing disease-causing non-native pathogens [7]. It is indispensable to identify, isolate, and culture native copepods as they can be comparatively more suitable as a live feed.

Among the copepods, harpacticoid species are more advantageous due to their smaller size, high nutritious profile, and easy food adaptability [8]. Though harpacticoid is one of the best choices as a live feed for aquafarmers, their protocol for large-scale production is still lagging. So, it becomes necessary to understand the taxonomy and biology of harpacticoid copepods in order to identify native species which helps to design the proper culture protocol for identified copepod. This chapter illustrates the procedure to isolate and identify harpacticoid copepods.

2 Materials

2.1 Collection of Zooplankton Sample

- Plankton net.
- Hand gloves.
- Habitat water.
- 10 L water can.
- Glass tank.
- Aerator.
- pH pen.

2.2 Isolation of Harpacticoid Copepod

- Plankton strainer.
- Gloves.
- Table lamp.
- Beaker 200 mL.
- Watch glass (big, medium, and small).
- Dissection microscope.
- Pasteur pipette.
- Fine brush.

2.3 Identification of Harpacticoid Copepods

2.3.1 Morphology

- 5% formaldehyde solution.
- 10% glycerol.
- 90% ethanol.
- Rose bengal (1 g/L).
- Watch glass (small).
- Pasteur pipette.
- Glass slides.
- Binocular compound microscope with camera.

2.3.2 Camera Lucida Drawings

- 5% formaldehyde solution.
- Watch glass (small).
- Pasteur pipette.
- Glass slides.
- Dissection set.
- Tungsten needle.
- Camera lucida.
- Compound microscope.
- Dissection microscope.

2.3.3 SEM Analysis

- Scanning electron microscopy analysis of the sample.

3 Methods

3.1 Collection of Zooplankton Sample

- Collect the zooplankton sample using a handled plankton net made up of bolting silk with 50 μm mesh size.
- Transfer the filtrate to the 10 L water can.
- Perform zooplankton sampling only during the early hours of the day.
- Carefully bring the collected zooplankton sample to the laboratory and transfer to a glass tank.

3.2 Isolation of Harpacticoid Copepod

- Filter the laboratory-maintained zooplankton samples using a plankton strainer.
- Transfer the filtrate to a big watch glass and keep it near the source of light.
- Based on their phototactic nature, harpacticoid copepods can be isolated from zooplankton samples.
- Use a fine brush or Pasteur pipette to transfer harpacticoids to a fresh medium or small size watch glass containing the filtered habitat water.

3.3 Identification of Isolated Harpacticoid Copepod

3.3.1 Morphology

- Transfer the fixed sample to a watch glass containing an ethanol and glycerol mixture (90% ethanol and 10% glycerol) containing Rose bengal (1 g/L).
- Wait until the alcohol evaporates.
- Place the specimens on a glass slide with the drop of glycerol and observe under the compound microscope.
- Photograph the structure of the whole animal.
- Identify the species with the aid of identification keys provided by taxonomists [9–12].

3.3.2 Camera Lucida Drawings

- Place the fixed specimen on the slide with the drop of glycerol.
- Dissect the body parts such as prosome, urosome, and appendages.
- Mount the dissected body parts.
- Attach the camera lucida to a compound microscope.
- Observe the mounted glass slide with the sample under a camera lucida fixed compound microscope.
- Observe and draw the impression of the sample that falls on the paper.
- Camera lucida drawings can be described following standard terminology [13].

3.3.3 SEM Analysis

- Fix and process the sample using the standard protocol.
- Photomicrograph the specimen at different angles and magnifications.

References

1. Gee JM (1989) An ecological and economic review of meiofauna as food for fish. Zool J Linnean Soc 96(3):243–261
2. Sampey A, McKinnon AD, Meekan MG, McCormick MI (2007) Glimpse into guts: overview of the feeding of larvae of tropical shorefishes. Mar Ecol Prog Ser 339:243–257
3. Arndt C (2013) Testing the suitability of harpacticoid copepods as food for marine fish larvae. Doctoral dissertation, Christian-Albrechts Universität Kiel
4. Hagiwara A, Lee CS, Shiraishi DJ (1995) Some reproductive characteristics of the broods of the harpacticoid copepod *Tigriopus japonicus* cultured in different salinities. Fish Sci 61(4): 618–622
5. Sun B, Fleeger JW (1995) Sustained mass culture of *Amphiascoides atopus* a marine harpacticoid copepod in a recirculating system. Aquaculture 136(3–4):313–321
6. Yasmeen MF, Saboor A (2016) The capability of harpacticoid copepod to ingest bacteria as food. Asian J Multidiscip Stud 4(13):41
7. FAO (2020) The state of world fisheries and aquaculture 2020. Sustainability in Action, Rome, p 190
8. Saboor A, Altaff K (2012) Demographic characteristics of *Onychocamptus bengalensis* (Copepoda: Harpacticoida)–a potential live feed for aquaculture. Revelat Sci 2(01):51–57
9. Bruno MC, Reid JW, Perry SA (2005) A list and identification key for the freshwater, free-living copepods of Florida (USA). J Crustac Biol 25(3):384–400
10. Gómez S, Gee JM (2009) On four new species of *Cletocamptus Shmankevich*, 1875 (Copepoda: Harpacticoida) from inland waters of Argentina. J Nat Hist 43(45–46):2853–2910
11. Lee W, Huys R (1999) New Normanellidae (Copepoda: Harpacticoida) from western Pacific cold seeps including a review of the genus Normanella. Cah Biol Mar 40(3): 203–262
12. Schizas NV, Shirley TC (1994) *Onychocamptus Yrusensterni* (Copepoda, Harpacticoida, Laophontidae) – a new species from Krusenstern Lagoon, Alaska. Crustaceana 66(2): 227–239
13. Huys R, Boxshall G (1991) The orders of copepods, Copepod evolution, vol 159. The Ray Society, London, pp 31–314

INDEX

John Thomas and Natarajan Amaresan (eds.), *Aquaculture Microbiology*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-3032-7,

M

N

P

Q

R

S

T

Z