Chapter 6

Detection of pathogenic of Vibrios: *Vibrio mimicus*, *Vibrio harveyi* and *Vibrio parahaemolyticus*

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Aquaculture is one of the growing vibrant food production sectors worldwide. However, the major drawbacks in this system are the sudden onset of diseases, especially by *Vibrio* spp. This is becoming a great concern in larval and juvenile penaeids. Hence, the monitoring of aquaculture environments for pathogenic Vibrios is essential to control the spread of Vibrio infections. The members of the genus *Vibrio* are the most important food-borne and aquatic pathogens which are responsible for illness in humans and cause large-scale mortality in the aquaculture sector. Nowadays in the international trade of marine fishes, testing of *Vibrio* species has become a criterion of microbiological testing. Even though *Vibrio* species are a common inhabitant of the aquatic environment, some species are emerging as pathogens which can cause up to more than 50% of deaths of all clinical cases. Major *Vibrio* sp. viz. *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. mimicus, and V. splendidus* are usually associated with shrimp diseases. *V. harveyi* is associated with luminescent vibriosis in shrimps e.g., *Litopenaeus vannamei* and *Penaeus monodon* and it is the most important etiological agent for mass mortality in *P. monodon*. The mode of infection in fish mainly consists of penetration of bacterium to the host tissue mainly by the chemotactic activity, followed by deployment of the iron sequestering system and eventually damages the fish through extracellular products i.e., hemolysin and protease.

Traditional method of detection of pathogenic *Vibrio* species

There are well-established isolation and biochemical confirmation procedures for pathogenic *Vibrio* spp. Which were described in ISO and BAM protocol for Vibrios. First stage in traditional detection methods exploits the ability of *Vibrio* species to grow rapidly at relatively high pH values. Media

containing sodium chloride and with a pH of about 8.6, such as alkaline saline peptone water (ASPW), are used for enrichment. Typically, a 6-hour preliminary enrichment (at 41.5°C for fresh products, or 37°C for frozen or salted products) is followed by a second enrichment in ASPW at 41.5°C (for *V. cholerae* and *V. parahaemolyticus*) or 37°C (for other species) for 18 hours. Preliminary identification based on colony appearance on TCBS agar is traditionally confirmed using classical biochemical tests. The second enrichment culture is inoculated onto thiosulphate citrate bile salts sucrose (TCBS) agar and one other optional selective medium and incubated at 37°C for 24 hours. On TCBS agar, *V. mimicus* colonies are green, *V. parahaemolyticus* colonies appear blue-green and *V. harveyi* colonies are green in color. Selective chromogenic agar media specifically designed for the differentiation of pathogenic *Vibrio* species are also available.

Protocol for the isolation of *V. mimicus* from fish

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Incubate APW at 35 ± 2 ^{0}C for 16 to 18 hours and transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate TCBS Plates overnight at 35 ± 2 ^{0}C

⇩

V. mimicus appears as small 2-3 mm, smooth green colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Gram negative short rods
- String test positive
- Arginine decarboxylase-Negative
- Lysine, Ornithine decarboxylase- positive
- Sucrose Negative
- Growth in 0% salt, no growth in 6% salt

Protocol for the isolation of *V. harveyi* from aquaculture samples

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Again, transfer a transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate overnight at 35 ± 2 ^{0} C

⇩

V. harveyi appears as large 2-3 mm, green colony

⇩

streak to marine agar and check for fluorescence

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation: These bacteria are

1. Gram negative, Motile rods
2. Oxidase- and catalase-positive
3. Green colonies on TCBS agar
4. These isolates do not utilize inositol, sorbitol, and melibiose
5. lysine decarboxylase, ornithine decarboxylase, and gelatinase-positive,
6. Sensitive to the vibrio static reagent, 0/129.
7. Glucosamine positive.

Protocol for the isolation of *V. Parahaemolyticus* from seafood

All the media used for the biochemical identification of *Vibrio parahaemolyticus* should contain 2 or 3% NaCl

25 g of Sample
(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 3% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. Parahaemolyticus appears as round, opaque, green or bluish colonies 2-3 mm in diameter on TCBS

⇩

Pick typical colonies on to TSA slants with 3% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation

- Oxidase positive
- Gram negative, straight/ curved rods
- Non H_2S producer
- Growth in 3 %, 6%, 8% NaCl, No growth in 0 % NaCl
- *V. parahaemolyticus* can be differentiated from other Vibrios by ONPG, Salt tolerance and lactose reactions
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129

Protocol for the isolation of *V. alginolyticus* from aquatic samples

All the media used for the biochemical identification of *Vibrio algionlyticus* should contain 2 or 3% NaCl

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 3% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. alginolyticus appears as large yellow colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 3% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive, Gram negative
- Growth in 3 %, 6%, 8%, 10% NaCl, No growth in 0 % NaCl
- Decarboxylase test results–Arginine negative, Ornithine positive, Lysine positive.
- Sugar test results- Sucrose, D-Mannose, D-Mannitol -Positive, D-cellobiose, Lactose, Arabinose –negative,
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129.

Protocol for the isolation of *V. vulnificus* from aquatic samples

All the media used for the biochemical identification of *Vibrio vulnificus* should contain 1-2% NaCl. *Vibrio vulnificus* is a waterborne bacteria which affect the vulnerable population with iron storage disorders. This bacteria can cause ulcerative disease in cultured fishes and can cause of mass mortality

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 2% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 2% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. vulnificus appears as 2-3 mm green colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Catalase positive
- Gram negative
- Growth in 1 %, 3%, 6%, NaCl, No growth in 0 % NaCl
- Decarboxylase test results–Arginine negative, Ornithine positive, Lysine positive for biotype 1. Lysine positive arginine, ornithine negative for biotype 2
- Salicin, mannitol, cellobiose, lactose, indole positive.
- Citrate, acetoin production and urease negative
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129.
- Resistant to colistin

Molecular methods

A simple and rapid identification method of *Vibrio* causing the disease to aquaculture settings is essential for taking preventive and curative measures in aquaculture. PCR-based identification is a suitable alternative because it is comparatively easy, less expensive, and can be completed within several hours. However, the success of this method depends on the selection of target genes, which should be species-specific, widely distributed, and also stable in the genome. PCR assays for the identification of pathogenic vibrio species are listed in table 1.

Table 1: PCR detection of pathogenic Vibrios

Pathogen	Primer name	Primer	PCR conditions & product size.	Product size
V. parahaemolyticus	*Species specific toxR-F* *toxR-R*	F:5'-GTCTTCTGACGCAATCGTTG-3' R: 5'-ATACGAGTGGTTGCTG	94°C, 03 min 30; 94°C for 01min 63°C for 1.5 min	368bp

		TCATG-3’	72^{o}C for 1.5 min 72^{o}C for 03 min	
	Virulence specific *tdh*	F**:**5’-TGACTGTGAACATTAATGA-3’ R**:**5’-CGATTCTTTGTTGGATATAC-3’	94^{o}C, 03 min 30; 94^{o}C for 30 sec 45^{o}C for 30 sec 72^{o}C for 01 min 72^{o}C for 05 min	263 bp
	Virulence specific *trh*	F**:**5’-TTGGCTTCGATATTTTCAGTA TCT-3’ R**:**5’-CATAACAAACATATGCCCATT TCCG-3’	94^{o}C, 03 min 30; 94^{o}C for 01min 58^{o}C for 01 min 72^{o}C for 01 min 72^{o}C for 03 min	500bp
Vibrio harveyi	*Tox R*	F:5’-GAAGCAGCACTCACCGAT-3’ R:5’-GGTGAAGACTCATCAGCA-3’	94^{o}C, 05 min, 30; 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min, 72^{0}C for 10 min	382 Bp
V. mimicus	*Vmh*	F:5’-GGTAGCCATCAGTCTTATCACG-3’ R:5’-ATCGTGTCCCAATACTTCACCG-3’	95^{o}C for 30 cycles; 95^{o}C for 45 sec 55^{o}C for 45 sec 72^{o}C for 35 sec	390 bp

			72°C for 05 min	
V.alginolyticus	*VAL*	F:5'-CGA GTA CAG TCA CTT GAA AGC C-3', R: 5'-CAC AAC AGA ACT CGC GTT ACC-3'	95° C for 15 min,30; 94°C for 30 s, 57°C for 30 s, 72°C for 60 s, 72°C for 5 min	737 bp
Vibrio vulnificus	*vvhA*	F-CGCCGCTCACTGGGG CAGTGGCTG R- CCAGCCGT TAACCGAACCACCCGC	95° C for 10 min,25 cycles 94°C for 60 s, 62°C for 60 s, 72°C for 60 s, 72°C for 5 min	518 bp

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