International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 29-09-2022, Accepted: 14-10-2022, Published: 31-10-2022
Volume 7, Issue 10, 2022, Page No. 162-165

Screening of virulent bacteria *Aeromonas hydrophila* from gastrointestinal tract of ornamental aquarium fish

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Abstract
Animals in the aquatic environment carry bacterial flora, which is a reflection of the flora in the environment. Intestinal bacteria, such as *Aeromonas sp*. and *Vibrio sp.* often cause opportunistic infections. Conventional approaches, such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic disease. Antibiotics are administered to aquariums primarily to prevent or treat bacterial diseases. The indiscriminate use of antimicrobials calls for a better understanding of the impact of these compounds on the microflora of the environment and the cultured fish. Virulence factors of *A. hydrophila*, which contribute to their pathogenicity, include the production of endotoxins, extra cellular enterotoxins, hemolysin, cytotoxins and protease, the ability to adhere the cells, and the possession of certain surface proteins. These virulent determinants, most of whose the mechanisms of action remain to be determined, are involved sequentially in enabling the bacteria to colonize, gain entry, establish, replicate and cause damage in host tissues and to evade the host defense system and spread, eventually killing the host. Current studies to analyze the gut micro flora of the ornamental aquarium fish *Carassius auratus* from infected freshwater ornamental fishes. The present study characterizes the virulence factors of the selected *A. hydrophila* strains.

Keywords: *A. hydrophila; Carassius auratus;* virulence; infections; micro flora

Introduction
Globally the ornamental fish culture is a powerful income and employment generating industry. In the aquaculture sector, ornamental fish breeding, culture and trade provide excellent opportunities as a non–food fishery activity for employment and income generation. It is environment friendly, socially acceptable and involves low investment for adopting as a small scale enterprise with high return. The attractive coloration and quiet disposition of ornamental fish provide a source of joy and peace for people irrespective of age group (Swain *et al*., 2007) [7].

Disease outbreaks are being increasingly recognized as a significant constraint on aquaculture production and trade, affecting the economic development of the sector in many countries. Infectious diseases in ornamental fish aquaculture are crucial factors which inhibit the expansion and socio-economic development. Bacterial disease is the most common infectious problem of ornamental fishes. Collectively, only water quality problems exceed bacterial diseases in the area of pet fish morbidity and mortality. The majority of bacterial infections are caused by Gram-negative organisms including the following pathogenic genera, *Aeromonas, Citrobacter, Edwardsiella, Flavobacterium (Flexibacter), Mycobacterium, Pseudomonas*, and *Vibrio. Streptococcus*, a Gram-positive genus, has been shown to cause disease in ornamental fishes (Inglis *et al*., 1993) [4]. Bacteria may be the primary cause of disease, or they may be secondary invaders, taking advantage of a breach in the fish's integument or compromise of its immune system (Khan *et al.*, 2013) [5]. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment, whether freshwater or marine. Nearly every bacterial pathogen of fish is capable of living independently away from the fish host. Virtually any extrinsic stress, including shipping, crowding, poor water quality, and inadequate nutrition, may predispose an ornamental fish to bacterial disease.

Aeromonas hydrophila is associated with aquaculture diseases and accounted for more than 50 % of the isolated aeromonads from crusian carp in Zeijiane province of china (Nielsen *et al.*, 2001). *A. hydrophila*, a Gram negative facultative anaerobic short bacillus, causes red fin disease, hemorrhagic septicemia, motile aeromonad septicemia and other infections in *C. auratus* (Biradar *et al*., 2007) [2]. In addition to *A. hydrophila*, bacteria which have been implicated in MAD include *A. sobria, A. caviae, and A. veronii.* These are ubiquitous organisms and opportunistic pathogens that take advantage of stressed and immune-compromised fishes.

Materials and Methods
1. Collection of infected ornamental Gold fish (*Carassius auratus*)
Infected fish samples were obtained from local ornamental aquariums in Azhagiyamandapam town of Kanyakumari District. Ornamental gold fish, *Carassius auratus* samples were used to isolate the *A. hydrophila* strains in the present work. All samples were analyzed on arrival otherwise they were stored at 4 °C until next day, for bacterial isolation.

2. Isolation of pure bacterial culture
The single colonies were streaked on to the nutrient agar slants were stored. This was further streaked on to the TCBS, Mac Conkey, EMB and Aeromonas isolation agar plates by using the quadrant streaking methods then the plates were incubated. After incubation the colonies

appeared in green and yellow colour were picked up and inoculated on to the nutrient for further studies. The colonies were also streaked onto the Aeromonas isolation agar. After incubation the green colour colonies were appeared as like as Vibrios on the TCBS agar plates. Then these colonies were inoculated onto the nutrient broth for further studies.

3. Biochemical confirmation

The biochemical tests were conducted to determine the physiological characters, carbohydrate metabolism and production of nitrogenous compounds. The characters such as capsule formation, motility and formation of flagella were recorded under morphological studies. The physiological characters included methyl red (MR) and voges-proskauer (VP) medium; different enzyme hydrolysis and amino acid decarboxylase tests. Observations on carbohydrate metabolism reaction included production of acid and gas in glucose and galactose, production of acid in maltase, D- mannose (West and Colwell, 1984) [8].

4. Growth characteristics studies for identified gut micro flora

4.1. Effect of different media, pH and temperature

The selected strains such as *V.harveyi, E.coli* and *Aeromonas* were grown in different cultured media such as nutrient broth, Zobell marine broth, Tryptone soy broth and alkaline peptone water. The growth curves were measured till the cultures were reaches the decline phase. The selected strains were grown in Trypton Soy Brothat different pH level. The curves were measured till the cultures were reaches the decline phase. The selected strain *Vibrio harveyi, E.coli* and *Aeromonas* were grown at different incubation temperature which were inoculated onto the Tryptone soy broth. The growth curves were measured till the cultures were reaches the decline phase.

5. Enzymatic Screening

5.1. Screening of lipolytic activity

A preliminary qualitative analysis for lipolytic activity was conducted by using spirit blue agar plates. The spirit blue agar media was sterilized. After sterilization 1% of tyrosine was poured into the sterilized agar media. Shake well and poured into the sterile Petri plates. After solidification of the plates a single streak of tested organism was made on the agar plates. After incubation zone formation was not appeared around the tested strain.

5.2. Screening of cellulase activity

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria was grown on CMC agar containing (g/l) KH_2Po_4 1.0, $MgSO_4.7H_2O$ 0.5, Nacl 0.5, Feso4 0.01, MnSo4 0.01, NH_4NO_2 0.3, CMC 10.0, agar 12.0. The pH was adjusted to 7.0 with 1m NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulose. at the end of incubation, the agar medium was flooded with an aqueous solution was then poured off, and the plates were further treated by flooding with 1m NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated the selected strain produce the enzyme cellulose.

6. Antibiotic sensitivity studies for gut micro flora

The selected strains were inoculated on to the nutrient broth. After incubation of 24 hours these strains were swabbed on to the Muller Hinton Agar plates. For these eight antibiotics were used such as Chloramphenicol, Streptomycin, Neomycin, Gentamycin, Oxytetracycline, Methicilline, Ampicillin, and Amoxicillin. The sterile antibiotic discs were placed on to the plates. Then these plates were incubated at room temperature. After incubation the zone was appeared around the discs in the plates were measured.

Results

1. Isolation and biochemical confirmation of gut micro flora from *C. auratus*

Fifteen types of colonies were isolated from the gut of *C. auratus*. Among the fifteen colonies from the gut of *C. auratus* samples, dominant colonies were selected based on morphological and biochemical confirmative tests. The colonies are white to dirty white in colour. In that some are rhizoidal and some are mucoidal in nature. The colonies are 2to 5mm in diameter. The major bacterial genera are isolated from gut of *C. auratus* and their morphological and biochemical confirmation tests were performed and the results are given in the Table 1. The details of the isolation of the bacterial genera in the gut revealed that, there were four major bacterial such as *Vibrio harveyi, Aeromonas and E.coli* and were confirmed.

2. Growth characteristics of the gut micro flora

2.1. Different media on growth

The selected sp such as *V.harveyi, E.coli* and *Aeromonas* were grown in the medium such as Nutrient broth, Tryptic Soya broth and Alkaline peptone water. The growth rate were record upto the cultures were reaches the decline phase. The highest growth rate was observed at the tryptic Soya broth around 14, 16, 18 & 20 hours for the strains such as *V.harveyi, Aeromonas* and *E.coli* (Figure 1).

2.2. Different pH on growth

The selected strains were inoculated onto the tryptic soya broth at different pH level such as 6.5, 7.5, and 8.5 for *V.harveyi, E. coli* and *Aeromonas*. The pH level 3.5, 6, 9.5 for *Aeromonas* (Figure 2). Among the three pH values 6 pH range got maximum value for *E.coli* around 14 hours. Among the three pH values 8.5 pH range got maximum value for *V.harveyi* around 18 hours.

2.3. Different temperature on growth

The selected strains were inoculated onto the Tryptic Soya broth and incubated at different temperature such as 30, 35 and 40°C. Among the different incubations maximum growth rate was observed for *E.coli* at 30 around 10 hours. The maximum growth rate for *V.harveyi* at 40°C around 16 and 18 hours. For *E.coli* culture growth at 30°C around 16 hours and the growth of *Aeromonas* at 30°C around 12 (Figure 3).

3. Enzymatic screening

The screening test for lipase production was conducted by using the spirit blue agar. There is no zone formation was found around the strains. This shows that the negative result for lipase production (Fig 4). The screening test for cellulose production was conducted by using the CMC Agar. There is no zone formation was found around the

strains. This shows that the negative result for cellulose production (Fig 4).

4. Antibiotic sensitivity studies for gut micro flora

The antibiogram studies of the selected strains isolated from the gut of *C. auratus* against the selected Antibiotics (zone of inhibition in cm) are given in the Table 2. For these, six antibiotics were used such as Chloramphenicol, Streptomycin, Neomycin, Gentamycin, Oxytetracycline, Ampicillin. Among these antibiotics the maximum values were got for all strains by using the antibiotic Chloramphenicol at 1.5 cm in diameter. The minimum values for *E. coli* were got by using the antibiotic Neomycin, and Gentamycin. The minimum values for *V. harveyi* were got at 0.5 cm by using the antibiotic ampicillin. The minimum values for *Aeromonas* at 0.5 cm were got by using the antibiotic Neomycin.

Discussion

Culture of *C. auratus*, white shrimp is one of the most profitable ventures in aquacultures sector in India and in many countries of the world. Water plays an important role in the transmission of pathogenic organisms. These pathogenic agents enter water bodies through agricultural, domestic waste water or animal and human waste. Most pathogenic bacteria transmitted by water include *Aeromonas sp*, *Vibrio sp*, *Campylobacter sp*. and *Yersinia sp* (pianetti *et al*., 1998) [6].

The present study, there are around three types of colonies were isolated from the gut of *C. auratus.* Among the fifteen colonies from the gut of *C. auratus* samples, dominant colonies were selected based on morphological and biochemical confirmative tests. The dominant colonies are identified to *V. harveyi*, *Aeromonas sp* and *E. coli*. Even though the pathogenic bacteria isolated from the gut there is no virulence at the time of isolation. The protease, lipase and cellulase production were performed all isolates. Among the different strains *V. harveyi* had the higher protease activity and it revealed that, alkaline serine protease is one of the virulence factors and not significance in the production of lipase and cellulose activity.

The gut microbiota play important roles in extracting nutrients from the diet, Regulating host fat storage stimulating intestinal epithelium renewal, and directing the maturation of the immune system (Fenchel and Kofoes, 1976) [3]. Keeping these communities in balance is most likely crucial for health maintenance, and perturbation of microbial composition has been hypothesized to be involved in a range of diseases, within and outside the gut (Asfie *et al*., 2000) [1]. These opportunistic pathogenic microbes apparently establish lethal infections as a result of other primary conditions that might include other infectious diseases, nutritional diseases, Extreme environmental stress and wounds. Infections by these bacteria display massive colonization of the appendages and foregut followed by infection of the mid gut, hepatopancreas and a terminal septicemia.

Table 1: Morphological and biochemical confirmation test of the gut micro floral isolates from the gold fish *C. auratus*

S. No	Biochemical Tests		Isolated strains		
			1	2	3
1.	Gram staining		-	-	-
2.	Motility		+	+	+
3.	Oxidase		+	+	+
4.	Catalase		+	+	-
5.	Indole		+	+	-
6.	Methyl red		-	+	-
7.	Voges proskauer		-	+	-
8.	Citrate		+	+	+
9.	Arginine decarboxylase		+	+	+
10.	Lysine decarboxylase		+	+	+
11.	Ornithin decarboxylase		+	-	+
12.	Gelatin		+	+	-
14.	Nitrate reduction		+	+	+
15.	Carbohydrate fermentation	Acid	+	+	+
		Gas	-	-	-
1.	Glucose		+	+	+
2.	Galactose		+	+	+
3.	Mannose		+	+	+
4.	Maltose		+	+	+

1. *V. haryi*, 2. *Aeromonas* and 3.*E.coli.*

Table 2: Antibiogram studies against the gut microflora isolated from *C. auratus* using the different commercial antibiotics

S. No	Antibiotics used	Zone of inhibition (cm) against Gut micro flora		
		E. coli	***V. harveyi***	***Aeromonas* sp**
1	Chloramphenicol	1.3 ± 0.16	1.5 ± 0.04	1.5 ± 0.08
2	Streptomycin	0.8 ± 0.16	1.2 ± 0.08	1.0 ± 0.24
3	Neomycin	0.5 ± 0.04	1.15 ± 0.24	0.5 ± 0.02
4	Gentamycin	0.5 ± 0.02	1.2 ± 0.04	0.7 ± 0.122
5	Ox tetracycline	0.8 ± 0.16	1.0 ± 0.044	0.7 ± 0.004
6	Ampicillin	0.5 ± 0.032	0.5 ± 0.022	1.0 ± 0.06

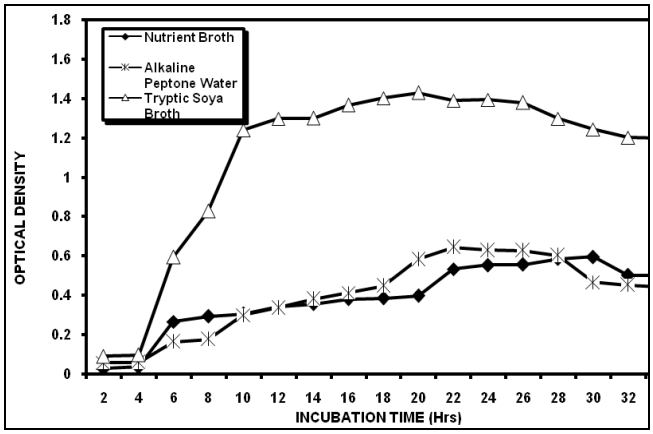

Fig 1: Growth characteristics of *Aaeromonas sp* isolated from the gut of *C. carpio* cultured in different growth media

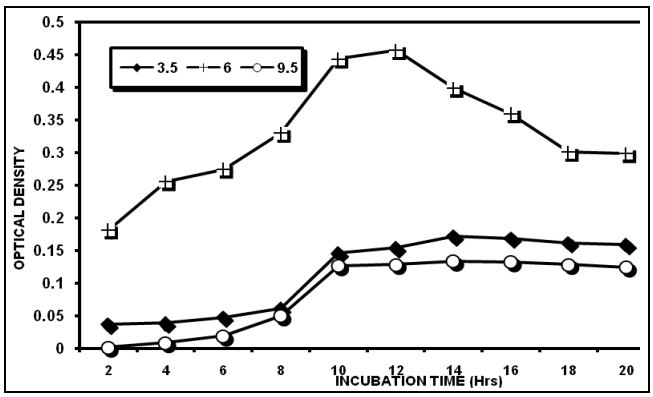

Fig 2: Growth characteristics of *Aeromonas sp* isolated from the gut of *C. auratus* cultured in different pH

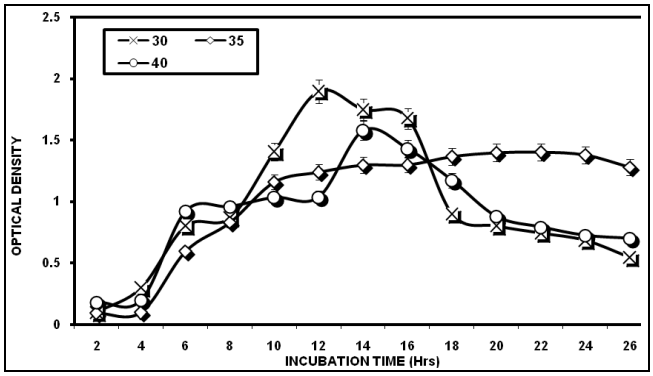

Fig 3: Growth characteristics of *Aeromonas sp* isolated from the gut of *C. auratus* cultured in different temperature incubation

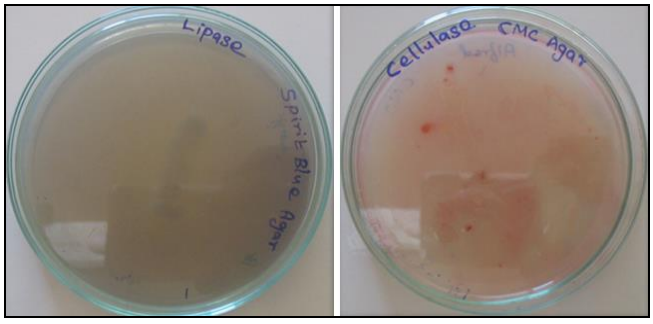

Fig 4: Lipase and Cellulase activity of the gut micro flora isolated from *C. auratus*

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