Species diversity and antibiotic sensitivity of pathogenic bacteria isolated from hatchery bred shrimp larvae in Sri Lanka

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Abstract

The larval stages of shrimps are highly prone to bacterial diseases and in order to prevent disease losses, antibiotics are indiscriminately used in hatcheries of Sri Lanka. Considering this situation, a study was undertaken in shrimp hatcheries to identify the common bacterial pathogens and the efficacy of antibiotics commonly used on these pathogens.

From a total of 96 bacterial isolates regarded as pathogenic, 39 were found to be gram negative species, which are generally considered as being pathogenic to shrimps. Of these, 20 isolates were identified as *Vibrio alginolyticus, V. fluvialis,* and *Pseudomonas aeruginosa*. *V. alginolyticus* was found to be the most prevalent species at a level of 85%, while others were present at 10 and 5% levels, respectively. Oxytetracycline and Erythromycin were the commonly used antibiotics in hatcheries. In vitro antibiotic sensitivity testing showed that all the pathogenic bacteria were sensitive to Oxytetracycline with 20% isolates showing high sensitivity and 80% intermediate sensitivity. Sensitivity to Erythromycin was low and while none of the isolates was highly sensitive, 29% showed intermediate sensitivity. Results of this study highlight an alarming trend, namely, that of low effectiveness of antibiotics used against Vibriosis in shrimps.

These results further suggest the importance of using alternative methods for controlling Vibriosis, such as improved environmental and nutritional management practices. The development of such practices are likely to be the best way forward in dealing with disease and environmental problems on prawn farms, in preference to the indiscriminate use of antibiotics.

Keywords: Diseases, Bacterial isolates, Antibiotics

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Introduction

In Sri Lanka, *Penaeus monodon* (Black Tiger shrimp) is an important commercial species cultured both for its food value as well as for export. *P. monodon* culture is carried out mainly in the Western and Northwestern coastal areas where environmental conditions are most favourable for their growth (FAO, 1997). The shrimp farming industry in Sri Lanka is dependent entirely on hatchery-bred post-larvae (PL). The quality of shrimp larvae is, therefore, considered a key factor in influencing successful shrimp culture in Sri Lanka and good hygiene is of paramount importance in hatcheries to ensure the production of good quality PLs (FAO, 1997).

Bacterial, viral, fungal and parasitic diseases are the major causes of farm mortality and production losses in culture systems in hatcheries (Brock and Master, 1992). Bacterial diseases can cause a range of problems in hatcheries leading to growth retardation as well as mass and sporadic mortalities (Karunasagar *et al.,* 2004). Although presently more attention is been given to the control of viral diseases, bacterial diseases are also as important in the management of these farms.

Microflora associated with larval stages of shrimp affects the health and development of the larvae. Common bacterial diseases of shrimps express as both cuticular and systemic infections. The bacterial genera implicated in these infections are known to be present as part of the normal gut microflora as well as in the water column and tank walls of commercial hatcheries. These bacteria, however, are usually opportunistic and often become pathogenic only when the management is poor (New *et al.*, 2010).

Diseases caused by *Vibrio* species and to a lesser extent several other gram–negative genera, are of considerable importance to marine shrimp farming worldwide (Brock and Master, 1992). *Vibrio* epidemics are a frequent problem in shrimp hatcheries (Boonyaratpalin, 1990). The principal bacterial species identified from epidemics of bacterial diseases in marine shrimp are *V. anguillarium, V. alginolyticus, V. cholerae, V. damsela, V. harveyi, V. parahaemolyticus, V. splendidus, V. vulnificus, Aeromonas* spp. and *Pseudomonas* spp. (Brock and Master, 1992).

In Sri Lanka, Luminescent Vibriosis has been recognized as the most serious disease affecting hatchery production of *P. monodon* and has been found to cause up to 100% mortality of *P. monodon* in the zoea, mysis and post-larval stages. Luminescent strains of *Vibrio* species have been implicated in outbreaks of shrimp hatchery bacterial diseases mainly in the Northwestern coastal areas of Sri Lanka (Wijegoonawardena and Siriwardena, 1996).

Antibiotics are commonly used to control the bacteria populations in shrimp hatcheries. However, these antibiotics are applied in an *ad hoc* manner with consequences leading to alteration of microbial communities and the generation of drug-resistant strains of bacteria (Subasinghe *et al.,* 1996). The phenomenon of drug resistance can be transferred to pathogenic bacteria and lead to a reduced efficacy in the antibiotic treatment of diseases caused by such resistant pathogens. There is little information on the quantities of different chemotherapeutics used in the shrimp farming industry in Sri Lanka, but the reported increase in their use has caused concern on the adverse impacts on human health and the environment.

This study was carried out to identify the common bacterial pathogens in our hatchery systems and to determine the management practices that can effectively control the disease conditions caused by these pathogens.

Materials and Methods

Study area and data collection

The study was carried out in the Northwestern and Western coastal belt in Sri Lanka where the majority of the shrimp hatcheries are located, and covered the Negombo, Puttalam and Chilaw areas. All the hatcheries operating in these areas were included in the study. Basic background information relating to each of these hatcheries was collected through a questionnaire-based survey.

Sampling Criteria

The hatcheries were visited biweekly and PLs were collected for bacteriological analysis with the help of strainers which had been sterilized by spraying with 70% ethanol.

The body surfaces of the collected PLs were also sterilized in the same manner and immediately washed with sterile sea water. Surface sterilized PLs were placed in sterilized bottles containing sterile sea water, overlaid with a layer of sterile paraffin oil to minimize contamination and transported to the laboratory under cool conditions.

Isolation and identification of bacteria

In the laboratory, the collected samples were crushed with a sterile inoculation loop and the exudates streaked on Tryptic Soy Agar (TSA) plates which were incubated at 35°C for 48 hours. Based on the colony shape and morphology, the most dominant colony type in each plate was picked up and isolated as a pure culture. Gram staining was carried out on the isolated pure cultures. Since all the known pathogenic bacteria of shrimps are grouped under either gram-negative rods or and gram- positive cocci, only such bacterial isolates were taken for further identifications. Selected bacterial strains isolated from the diseased shrimp were identified with the help of biochemical test series according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (2002), and confirmed using the tests incorporated in API 20E miniaturized identification test strips (Bio Meriux, France).

Antibiotic sensitivity test (AST)

The Kirby-Bauer disc diffusion technique was used to study the antimicrobial susceptibility (Bauer *et al.,* 1966). The turbidity of the bacterial suspension used for the AST was standardized according to the McFarland's nephelometer standards Number 5. The diameter of inhibition zones of the each tested antibiotic disk was measured and the results were interpreted as sensitive, intermediately sensitive or resistant for each antibiotic disk based on the National Committee for Clinical Laboratory Standards (NCCL). Sensitivity was tested against commonly used Antibiotics, namely, Oxytetracycline, Erythromycin, Naldixic acid, Tetracycline and Amoxicillin.

Results

Post larvae from 27 hatcheries were studied. Ninety six bacterial cultures were isolated of which 39 isolates were suspected to be pathogenic while 20 isolates were identified as known shrimp pathogens. Those 20 strains belonged to the genera *Vibrio* and *Pseudomonas* which are responsible for Vibriosis and Bacterial septiceamia of shrimps,

Table 1. Morphological and biochemical characteristics of bacterial isolates from post larvae of *Penaeus monodon* hatcheries

Test	*V. alginolyticus*	*V. fluvialis*	*P. aeruginosa*
Gram stain	-	-	-
Shape	R	R	R
Motility	+	+	+
Cytochrome oxidase	+	+	+
Catalase	+	+	+
O/F test	F	F	O
Acid production from Carbohydrates			
Glucose	V	+	+
Sucrose	+	+	-
Lactose	-	-	-
Mannitol	+	+	+
Maltose	+	+	+
Salicin	+	+	-
Xylose	-	-	+
Gas from glucose	-	-	-
H_2S production	-	-	-
Citrate utilization	+	+	+
Aesculin hydrolysis	-	-	-
Gelatinase	-	+	+
Nitrate reduction	+	V	+
Methyl red test	+	+	+
Voges Proskauer test	+	-	-
Indole production	+	V	-
Decarboxylation of			
Arginine	-	+	+
Lysine	+	-	-
Ornithine	+	-	-
Growth at			
20 to 30ºC	+	+	+
37ºC	-	-	-
42ºC	-	V	-
NaCl tolerance			
00%	-	-	NT
02%	+	+	NT
04%	+	+	NT
06%	+	+	NT
08%	-	V	NT
10%	-	-	NT
TCBS agar	Y	Y	-

+ = Positive; - = Negative; NT = Not tested; F = Fermentative; O = Oxidative;
R = Rods, Y = Yellow; G = Green, V = Variable among strains, Y = Yellow colonies.

respectively. They were identified as *V. alginolyticus, V. fluvialis,* and *Pseudomonas aeruginosa* based on various morphological, physiological and biochemical tests as shown in Table 1. *V. alginolyticus* was found to be the most prevalent species at a level of 85%, and others at 10% and 05% levels, respectively.

In respect to the geographical distribution of the isolated pathogenic bacteria, *V. alginolyticus* was found along the Northwestern coastal area and most frequent in Ambakandwilla while *V. fluvialis* and *P. aeruginosa* were limited to the Ambakandawilla area. Some background data on the selected hatcheries and the presence of isolated pathogens are summarized in Table 2.

Table 2. Location, stock density, bacteria isolated and the Chemotherapeutic used in different tanks

Bacterial pathogen	Location of brooders Collected	PL stage	Stocking density (PLs/ One tone tank)	Chemotherapeutic used
V. alginolyticus	Not known	12	150,000	Triplane, EDTA
P. aeruginosa	Not known	18	50,000	Oxytetracycline Triplane, EDTA
V. alginolyticus	Beruwela	05	100,000	Terramycin/ Oxytetracycline, Triplane, EDTA
V. alginolyticus	Wennappuwa & Kaluthara	15	150,000	Oxytetracycline Triplane, EDTA
V. alginolyticus	Beruwela	05	100,000	Terramycin/ Oxytetracycline, Triplane, EDTA
V. alginolyticus	Beruwela	18	150,000	Oxytetracycline Triplane, EDTA
V. alginolyticus	Beruwela	05	150,000	Tetramycine Oxytetracycline, Triplane, EDTA
V. alginolyticus	Beruwela	18	100,000	Erythromycin, Triplane, EDTA
V. alginolyticus	Beruwela	10	100,000	Triplane, EDTA
V. alginolyticus	Not known	20	Not known	Not known

V. fluvialis	Not known	18	50,000	Oxytetracycline, Tetracycline Triplane, EDTA
V. alginolyticus	Pothuwil	03	100,000	Erythromycin, Oxytetracycline Triplane, EDTA
V. alginolyticus	Walachchanei	12	Not known	Erythromycin, Oxytetracycline Triplane, EDTA
V. fluvialis	Walachchanei	06	50,000	Erythromycin, Triplane, EDTA
V. alginolyticus	Not known	18	50,000	Triplane, EDTA
V. alginolyticus	Not known	01	Not known	Erythromycin, Triplane, EDT
V. alginolyticus	Not known	20	Not known	Oxytetracycline, Tetracycline,EDTA
V. alginolyticus	Chilaw	02	Not known	Erythromycin, Triplane, EDT
V. alginolyticus	Chilaw	13	50,000	Erythromycin, Oxytetracycline Triplane, EDTA
V. alginolyticus	Chilaw	05	50,000	Terramycin Oxytetracycline, Triplane, EDTA

Antibiotics have been used in these hatcheries both for prophylaxis as well as for treatment of infected shrimps. The antibiotics used were Oxytetracycline (Terramycin) and Erythromycin and of these, Oxytetracycline was the most commonly used antibiotic for treatment.

As shown in Table 3, drug sensitivity tests revealed that among the antibiotics used in the hatcheries, all the identified pathogenic isolates were sensitive to Oxytetracycline (100%).

Table 3. Sensitivity of bacterial isolates from shrimp hatcheries to various antibiotics

	Drug Sensitivity of bacterial isolates								
	V. alginoliticus			***V. fluvialis***			***P. aeruginosa***		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Oxytetracycline (30µg/disk)	03	14	-	01	01	-	-	01	-
Erythromycin (15µg/disk)	-	05	12	01	-	01	-	-	01
Amoxicillin (30µg/disk)	-	-	17	-	-	02	-	-	01
Tetracycline (30µg/disk)	01	05	11	01	01	-	-	-	01
Naldixic acid (30µg/disk)	-	02	15	01	01	-	-	-	01

S = Sensitive **I** = Intermediate sensitive **R** = Resistant

However, sensitivity to Erythromycin was much lower with only 32% of the isolated pathogenic bacteria being susceptible to this antibiotic. Other than these two commonly used antibiotics, three others (Amoxicillin, Tetracycline and Nalidixic acid) were included in the drug sensitivity test. It was found that all the identified bacteria were resistant to Amoxicillin while only 35 % of identified bacteria showed sensitivity to Tetracycline and only 12% of identified bacteria showed sensitivity to Naldixic acid.

Discussion

The present study revealed that three species of pathogenic bacteria namely *V. alginolyticus, V. fluvialis,* and *P. aeruginosa*, were present in diseased and healthy shrimp post larvae in hatcheries. Wijegoonawardena and Siriwardena (1996) recognized luminescent Vibriosis as the most serious disease affecting hatchery production of *P. monodon* in Sri Lanka. Since then luminescent strains of *Vibrio* species have been implicated in outbreaks of shrimp hatchery bacterial diseases mainly in the Northwestern coastal areas of the country. This may be due to the seasonal variations and sea water conditions of the area and according to Wijegoonawardena and Siriwardena (1996) the high levels of luminescent *Vibrios* in the sea water of this area is due to high organic matter. According to Shilo and Yetinson (1979) luminescent *Vibrios* grow very well in eutrophic sea water.

However, Hisbi *et al*., (2000*)* in a similar study in Indonesia, reported the presence of three pathogenic bacterial species in shrimps (*V. alginolyticus, V. damsela, and V. harveyi*). It can be seen that only *V. alginolyticus* was common to the two studies. However, opportunistic species may be expected to vary from one geographical area to another and from one hatchery to another within a country as well as in different countries.

According to Jayasree *et al.,* (2000) the presence of *V. alginolyticus, V. paraheamolyticus, V. anguillarum* and *P. aeruginosa* were recorded in white spot disease infected shrimp in India. A similar study done by Karunasagar *et al.,* (1997) in India also reported the presence of four species *V. alginolyticus, V. cholera, V. mimicus, and V. harveyi* in white spot infected shrimp. However, these studies were done for shrimp in pond culture systems, infected with the White Spot Syndrome Virus (WSSV).

In this study also *Vibrio* was identified as the dominant genus of the bacterial flora isolated from shrimp larvae in hatcheries. Different bacterial genera have been associated with infections of penaeid shrimp larvae, but *Vibrio* species undoubtedly emerges as the most harmful pathogenic bacteria. *V. alginolyticus* and *V. fluvialis,* which were identified in this study were recorded as the Vibriosis causing bacteria in shrimp (Lightner, 1996; Brock and Lightner, 1990). The term Vibriosis refers to a disease condition associated with one or more bacteria of the genus *Vibrio.* It is responsible for mortality of cultured shrimp worldwide (Chen *et al*., 2000) and it was identified as a stress induced disease in shrimps. Environmental stress factors are known to suppress host resistance and promote viral and bacterial infections (Jayasree *et al*., 2000).

All the bacterial isolates identified during the present study showed susceptibility to Oxytetracycline when compared with the tested antibiotics. Even for this antibiotic, very few (20%) showed high sensitivity and most (80%) showed intermediate sensitivity. The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug resistant strains and multiple antibiotic resistance in bacteria, which ultimately results in transfer of resistance to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases

(Manjusha *et al*., 2005). On the other hand antibiotics and disinfectant chemicals like formalin, used in hatcheries, can create health hazards for the persons handling them as well as killing off non-targeted organisms if discharged into the main water body through effluents (Vijayakumaran, 1997). According to the antibiotic sensitivity test conducted, majority of bacteria isolated in this study showed resistance against antibiotics used in hatcheries. Acquired antibiotic resistance in bacteria is generally mediated by extra chromosomal plasmids and is transmitted to next the generation (vertical gene transfer) and also exchanged among different members of the bacterial population (Horizontal gene flow) (Manjusha *et al.*, 2005).

Conclusions and Recommendations

The present study concludes that *Vibrio* species are most prevalent pathogens in shrimp hatcheries and antibiotic application on control of Vibriosis in hatcheries has limited effectiveness due to the development of resistant bacterial strains in the environment. Hence prevention of disease through proper management of environmental stress factors as well as improving host conditions through good nutrition is more important. Therefore better management practices for shrimp farming to deal with disease and environmental problems are likely to be the best way and need to be developed further.

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