Control of pathogenic *Vibrio* spp in freshwater prawn *Macrobrachium rosenbergii* hatchery using bleaching solution based on active chlorine

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A Thesis

By

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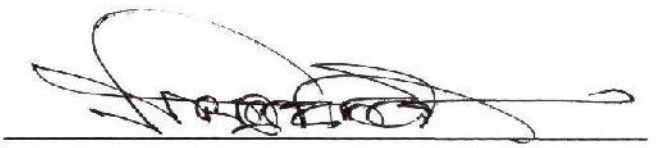
Approved as to style and content
By



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Declaration

Control of pathogenic *Vibrio* spp in freshwater prawn *Macrobrachium rosenbergii* hatchery using bleaching solution based on active chlorine

The results presented in the thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.

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My Respectable Parents

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All the praises goes to the almighty Allah for blessing me to complete my thesis for the degree of M.Sc. in Fisheries from Khulna University under the Fisheries and Marine Resource Technology Discipline.

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Table of Contents

List of Tables

List of Figures

05	Fig 5	a	Zone of inhibition produced by disc contained Oxytetracycline (12.5 ppm) against *Vibrio* was examined by slide calipers	23
		b	Zone of inhibition produced by disc contained Ciprofloxacin (10 ppm) against *Vibrio* was examined by slide calipers	23

CHAPTER ONE

INTRODUCTION

1. Introduction

Culture of shrimp and prawn plays a central part in the fisheries sub-sector in Bangladesh. It alone contributes more than 70% to the total export earnings from all the agro-based products (DoF, 2011). Presently brackish water shrimp farming has become more commercially preferred, with heavy reliance on single shrimp species of which the black tiger shrimp (*Penaeus monodon*), known as bagda, is the most important. On the other hand, culture of freshwater prawn known as golda (*Macrobrachium rosenbergii*) is not restricted to the coastal regions and is expanding at a rate of 10–20 per cent per annum, which currently occupies about one fourth of the total area (Biswas and Finan, 2004). In Bangladesh, a *Macrobrachium rosenbergii* hatchery first started in Cox's Bazaar in 1986 (Angell, 1994; Winrock International, 2007). By 1990, prawn farming had been extended to other southwest districts, such as Khulna, Satkhira and Jessore, owing to the development of export markets with high demand (Abedin *et al.* 2001). Since then it has become one of the most attractive investment opportunities in these areas (Ahmed 2001). Unlike tiger shrimp, the freshwater prawn is the economic mainstay of small-scale gher farmers, which is apparently better off in terms of negative environmental and social consequences (Ahsan, 2010). The construction of freshwater ghers has increased by 10-20 percent per annum (Abedin *et al.*, 2001) and is now the primary livelihood strategy of more than 100,000 rural households in the region (Biswas and Finan, 2004). However, a close assessment of the export trend over the last couple of years reveals that the growth was not always steady (Ahsan, 2013), due apparently to a number of challenges prevailing in the sector. In case of freshwater prawn industry, mass mortality of larvae and post larvae in hatcheries has been considered as the most crucial issue at the moment for the sustainability of this important sub sector.

Up until recently, freshwater prawn has been considered as less prone to disease than its marine counterpart, tiger shrimp. However, mass mortalities of larvae and post larvae in prawn hatcheries consecutively for the last few years have been turning many prawn hatcheries into total bankrupt. Over the past two years, since early 2011 however, serious production problems have affected most of the Bangladeshi prawn hatcheries, forcing them to slow down operations, or in many cases close down, at least temporarily (Dr. Matthew Briggs, 2013). Mortalities of prawn larvae due to infectious diseases must have been encountered sporadically since long but a systematic research on this problem is yet to be undertaken. This brings the whole freshwater prawn hatcheries on the verge of collapse in

one hand, and on the other hand increasing the pressure on wild for collecting PL despite stringent measures by government officials to ban wild fry collection.

It is known that a complex interplay of environmental factors, microbiological properties and management practices influence the success of the production cycle of prawn hatcheries. Apart from lack of ascetic condition in the hatchery premises, the quality of incoming and circulating waters greatly increase the number and population of bacteria in hatchery. Bacteria diseases have been implicated to be one of the most devastating diseases which can completely destroy hatchery productivity for extended periods (Suwanto *et al.*, 1998 and Lavilla-Pitago *et al.*, 1998). For a successful hatchery operation, it is crucial that the content of certain bacteria, particularly the pathogenic *Vibrio* spp. must be maintained to the minimum level possible.

Vibrios are ubiquitous in marine and estuarine environments and are commonly present in or on shellfish and other seafood. They are present in the environment as free-living and associated with different substrata (Tamplin *et al.*, 1990). The genus *Vibrio* includes gram-negative, rod or curved rod -shaped, facultative anaerobes, bacteria (FDA, 1992). Vibriosis is a term for several fish diseases causing serious problems for a wide range of wild and farmed species (Damsgaurd *et al.*, 2004). Luminescent bacterial diseases caused by members of the genus *Vibrio* are often the limiting factor in shrimp and prawn culture operations worldwide (de la Pena *et al.*, 2001). Larval prawns are particularly susceptible to *Vibrio harveyi* succumbing to what has been termed luminescent bacterial disease (Lavilla-Pitogo *et al.*, 1990). Additionally, there is an increasing list of aquatic animals for which *Vibrio harveyi* has also been reported as a major pathogen, including finfish (Balebona *et al.*, 1995, Saeed 1995), bivalves (Pass *et al.*, 1987) and phyllosoma larvae of the lobster *Jasus verreauxi* (Diggles *et al.*, 2000). Most of these vibrios secrete enterotoxins in foods, water or in the gastrointestinal tract (Nishibuchi and Depaola, 2005). *Vibrio harveyi* appears to release exotoxins (Liu *et al.*, 1996). Serious mortalities in *Macrobrachium rosenbergii* hatcheries caused by Luminescent bacterial disease due to *Vibrio* spp (Sharshar et al., 2008). *Vibrio parahaemolyticus* is part of the natural estuarian microflora and coastal marine waters and can be present in seafood, especially shellfish and bivalve mollusks (DePaola *et al.*, 2003; Zorrilla *et al.*, 2003). *Vibrio* species are part of the natural microflora of wild and cultured shrimps (Sinderman, 1990) and become opportunistic pathogens when natural defense mechanisms are suppressed (Brock and Lightner, 1990). It is thus imperative that the

pathogens especially *Vibrio* spp. in prawn hatchery system must be controlled for a successful hatchery operation.

Different techniques have been developed to control luminous bacteria in shrimp and prawn hatcheries including various antimicrobial agents amongst which hypochlorite-based bleaching powder solution is the most commonly used disinfectants for water disinfection in hatcheries. It can be applied for the deactivation of most microorganisms and it is relatively cheap. Bleaching powder is used in most prawn hatcheries in the form of calcium hypochlorite ($Ca(ClO)_2$) for water disinfection. When done correctly, chlorination can kill possible wild vectors of disease in hatchery. Usually, bleaching powder is used in hatcheries on the basis of recommended doses mentioned in the commercial container. However, it should be based on active chlorine. The term "active chlorine" is used because most commercial bleaches contain substantial amounts of chlorine in the form of chloride ions, which have no bleaching properties. It is actually the active chlorine that inhibits the bacterial growth and/or kills the bacteria, not the bleaching powder itself. This is why high bacterial load are often found from waters sampled from hatcheries where bleaching powder solutions are regularly used for general cleaning purposes (unpublished observation). It is, therefore, necessary to isolate and characterize *Vibrio* spp. from hatchery samples and investigate the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of commercial bleaches based on the empirically determined active chlorine concentration. As different approved antibiotics are used in hatchery operation so it is also necessary to test the sensitivity of different antibiotics.

Objectives of the study

The objectives of the present study are:

- To determine the concentration of active chlorine present in different commercial brand of bleaching powder used in the prawn hatcheries;
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of active chlorine against total bacteria; and
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of active chlorine against total *Vibrio* spp.

CHAPTER TWO

LITERATURE REVIEW

2. Literature Review

While there are a quite number of previous studies that dealt with *Vibrio spp* in shrimp hatcheries, there are few studies about *Vibrio spp* in prawn hatcheries. And there is a dearth of knowledge clearly establishing the relationship between the mortalities of prawn larvae by pathogenic *Vibrio spp* and use of bleaching powder to inhibit it. The following are some representative studies that deal with related terms.

DoF (2010) published that, in 2008–2009, Bangladesh produced 26138 t of prawns from different water bodies, while 90% of this production (23597 t) was by aquaculture from southwestern region and the rest by capture mainly from inland and coastal rivers, beels (wetlands) and floodplains. It indicates the importance of prawn farming in our country.

DoF (2011) also published, Culture of shrimp and prawn plays a central part in the fisheries sub-sector in Bangladesh. It alone contributes more than 70% to the total export earnings from all the agro-based products.

Abedin *et al.* (2001) observed that, the construction of freshwater ghers has increased by 10-20 percent per annum.

Biswas *et al.* (2004) stated that, the primary livelihood strategy of more than 100,000 rural households in the region was freshwater ghers.

Ahsan (2010) studied that, the freshwater prawn is the economic mainstay of small-scale gher farmers, which is apparently better off in terms of negative environmental and social consequences.

Ahsan (2013) also stated that, a close assessment of the export trend of freshwater prawn over the last couple of years reveals that the growth was not always steady.

Suwanto *et al.* and Lavilla-Pitago *et al.* (1998) observed that, Bacteria diseases have been implicated to be one of the most devastating diseases which can completely destroy hatchery productivity for extended periods.

Damsgaurd *et al.* (2004) stated about Vibriosis. He stated that, it is a term for several fish diseases causing serious problems for a wide range of wild and farmed species.

Sinderman (1990) observed that, *Vibrio* species are part of the natural microflora of wild and cultured shrimps.

Sharshar *et al.* (2008) studied that, serious mortalities in *Macrobrachium rosenbergii* hatcheries caused by Luminescent bacterial disease due to *Vibrio* spp.

Jawahar *et al.* (2004) studied the abundance of luminous bacteria in commercial penaeid shrimp hatcheries.

Karunasagar *et al.* (1994) observed Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection.

Islam *et al.* (2008) stated that, the minimum inhibitory concentration (MIC) represents the concentration of antimicrobial at which there is complete inhibition of growth of organism. He stated the importance of determination of MIC of certain antibiotic to inhibit bacterial growth.

Alsina, M. *et al.* (1994) provided the biochemical tests for identification of *Vibrio* spp.

Mahon C. R. *et al.* (2011) described about Diagnostic microbiology in their Textbook of Diagnostic microbiology. Different types of diagnosis are in the book and that are very much helpful.

Petrus, *et al.* (2011) stated about Minimum Bactericidal Concentration (MBC). They showed the concentration of antimicrobial agents needed for MBC.

Carson *et al.* (1995b) describe the MIC as 'the lowest concentration which resulted in maintenance or reduction of inoculums viability' over a 24 h contact time.

Remmal *et al.* (1993) apply a similar definition but extend the incubation time to 24–48 h after the appearance of growth in the control.

Ellis A. E. (2001) observed the abundance of *Vibrio harveyi* in *Penaeus monodon.*

Ransangan, J. *et al.* (2013) stated the sensitivity of chloramphenicol to *Vibrio. Vibrios are* sensitive to this antibiotic.

Horowitz *et al.* (2003) suggested providing better environmental and biological conditions to the infected population to increase its ability to resist diseases.

Romero, J. et al. 2012) stated, Use of probiotics can be fruitful for prawn hatchery operation.

Samuelsen, (2006) reported that, Oxytetracycline (OTC), one of the most commonly used antibiotics in fish farms and hatcheries, is very poorly absorbed through the intestinal tract of fish. It has been estimated that 70-80% of OTC is intact in the feces. So antibiotics should be used with proper way that can minimize environmental pollution. Water should be treated before excretion into the natural water.

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and Methods

3.1 Time duration of the study

The study was conducted from April, 2014 to April, 2015.

3.2 Sample collection

Larval prawns (*Macrobrachium rosenbergii)* and treated water (from larval rearing tanks) samples were collected from local prawn hatchery.

3.3 Laboratory requirements

3.3.1 Instruments:

- Autoclave machine (ALP)
- Incubator (Vision)
- Centrifuge machine (Vision)
- Electric balance
- Petri dish, test tube, eppendrof tube, beaker, conical flask
- Hot plate and magnetic stirrer (Vision)
- Solution mixer, burette
- Bacteria isolating loops
- Refrigerator (Wisecryo and LG-Green life)
- Bio-safety clean bench (Vision)
- Micro-pipette, Tips and tips box
- Water distiller
- Spectrophotometer (HITACHI, U-2910)
- Vernier calipers (Shanghai, China)

3.3.2 Reagents:

For isolation of bacteria-

- Peptone water (OXOID, CM0009)
- Nutrient agar (HIMEDIA, M001-500G)
- TCBS agar (HIMEDIA, M189-500G)
- Nutrient broth (HIMEDIA, M002-500G)
- Distilled water.
- Detergent powder.
- Simmons citrate agar (HIMEDIA, M099-500G)

- Sodium chloride
- α-naphthol and KOH for Voges-Proskauer (VP) test

Reagents for determination of active chlorine –

- Bleaching powder
- Standard sodium thiosulphate ($Na_2S_2O_3$) solution (0.1N)
- 10% Potassium iodide (KI) solution
- Dilutes acetic acid
- Freshly prepared starch solution as indicator

3.4 Total plate count

Microbiological analysis-Total Plate Count (T.P.C) was done according to the Bacteriological Analytical Manual of United States Food and Drug Administration (USFDA, 2001). The study was conducted at the Biochemistry and Molecular Genetics Lab of Khulna University.

3.4.1 Media preparation

- Definite amounts of Plate Counting nutrient agar (7gm) were being accurately weighted.
- These would take in separate volumetric flask containing distilled water 125ml (half of required volume).
- Then the final volume (250ml) being adjusted by pouring additional distilled water.
- Then the volumetric flasks being kept in autoclave for sterilization. Autoclave has to be done at a temperature of 121°C for about 15 minutes.
- After autoclave the agar media have to keep in the bio-safety cabinet for 20-30min for cooling and then transferred in required number of petridishes by 25ml volume for preparing plates.

3.4.2 Sample preparation & dilution (water Samples)

1ml of sample water was taken especially from the each sample and was transferred to cotton plugged bottle containing 9 ml distilled water. The sample was mixed properly by shaking the bottle carefully. Then the total amount became 10 ml. This made 1st decimal dilution (o.1). In this way four dilution tubes with the dilution strength, 10^{-1}through 10^{-4} were made.

3.4.2.1 Sample preparation & dilution (prawn larvae Samples)

- prawn larvae were homogenized with tissue homogenizer

- Centrifugation 3000 rpm (rotation per minute)
- Collection of Upper layer with micropipette in eppendrof tube
- Mixture 0.1ml stock solution + 0.9 ml distilled water
- Four dilution tubes with the dilution strength, 10^{-1}through 10^{-4} were made.

3.4.3 Pour plating & incubation

Then these samples were poured onto the petridishes (plates) previously had nutrient agar. Then the plates with solidified agar were incubated at 37^0C for 24 hours in the incubator.

3.4.4 Plate counting

After incubation, the plates were analyzed. Analysis, in this case, means simply counting the entire colony forming units (CFU). To get reliable results with the spread plate method, only those plates were counted that produced between 30 - 300 CFUs. For ease of counting, the plates were marked into quadrants and count each quadrant separately.

3.5 Isolation of *Vibrio* spp.

ISO (ISO/TS 21872-1, 2007) method was followed. *Vibrio* spp. was isolated from the collected prawn larvae and water .The total process was done by the following steps for prawn larvae:

Collection of larval prawn
↓
Homogenized with tissue Homogenizer + centrifugation 3000 rpm
↓
Collection of Upper layer with micropipette in eppendrof tube
↓
0.1 ml stock solution + 0.9 ml of alkaline saline peptone water
↓
Incubation for 6 h ± 1h at 37°C
↓
1ml of culture + 10 ml alkaline saline peptone water
↓
Incubation for 18 h ± 1h at 41.5°C
↓
Inoculation in TCBS media for 24 h at 37°C
↓
Isolation and enumeration of *Vibrio spp.* colonies

Water sample was directly spread on TCBS media and incubated for 24 h at 37°C and then isolation was performed.

3.6 Biochemical tests

Different biochemical tests were performed using the identification matrix provided by Alsina and Blanch (Alsina M and Blanch AR, 1994).

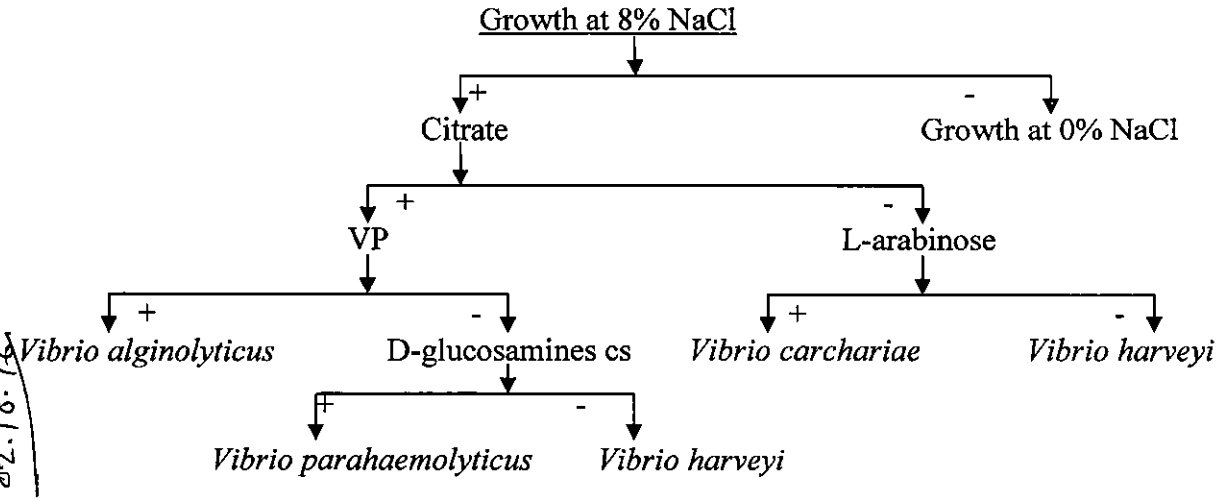

Fig1: Identification key for *Vibrio* spp

3.7 Stocking of cultured sample

After completing the biochemical tests the bacterial samples were stocked. The pure isolates were preserved in nutrient broth with 2% NaCl, in 30% glycerol and stored at -80 °C until use. (Ransangan *et al.* 2013)

3.8 Determination of active chlorine in bleaching powder sample

It was done by following way (http://www.google.com/url) at chemistry lab of Khulna University.

Procedure:

1 g of bleaching powder was accurately weighed and was transferred to a clean beaker. Then the powder was ground to a thin paste with water. Then the solution is made up to the 100 ml volume. 10 ml of the solution (use measuring flask) in a state of very fine suspension was taken into a conical flask and added 10 ml of distilled water into it. Then 10 ml of 10% potassium iodide (KI) solution and 10 ml of dilute acetic acid were added. This solution was titrated with standard sodium thiosulphate solution (taken in the burette) until the dark brown color changed to pale yellow. Then 2 ml of freshly prepared starch solution as indicator was

added, so the color of the solution turned into blue. The titration was continued till the disappearance of blue color of the solution and the volume of the titrant were noted down.

Calculations:

Normality of given bleaching powder solution (N_2) was calculated from Burette Readings. The following formula was done: $N_1 \times V_1 = N_2 \times V_2$

Where, Normality of standard $Na_2S_2O_3$ solution = N_1= 0.1N

Volume of standard $Na_2S_2O_3$ solution = V_1 =.....ml

Volume of given bleaching powder solution = V_2=.....ml

So, $N_2 = (N_1 \times V_1)/ V_2$

Amount of available chlorine = Normality of given bleaching powder solution (N_2) × Equivalent weight of chlorine = ...g/lit.

Amount of available chlorine present in 100 ml of the solution = (.....× 100)/1000 = ...g

The percentage of available chlorine present in the given sample of bleaching powder (BP) = (Amount of available chlorine/ weight of bleaching powder) × 100 = ... %

3.9 Antimicrobial susceptibility test

Susceptibility of bacteria to various doses of active chlorine was conducted. The following methods was done according to Mahon C. R. *et al.* (fourth edition), 2011. "Textbook of Diagnostic microbiology". These were-

3.9.1 Inoculum preparation and use of McFarland standards:

- **Inoculum Preparation:** Inocula was prepared directly by suspending colonies grown overnight on an agar plate.
- **McFarland turbidity standards:** A McFarland standard was prepared by adding specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a specific optical density. In this study the McFarland 0.5 standard was used, which contains 99.5 mL of 1% sulfuric acid and 0.5 mL of 1.175% barium chloride. This solution was dispensed into tubes comparable with those used for inoculum preparation, which were sealed tightly and stored in the dark at room temperature. The McFarland 0.5 standard provided turbidity comparable with that of a bacterial suspension containing approximately 1.5×10^8 CFU/mL.
- **Inoculum standardization:** To standardize the inoculum, the inoculated suspension (colonies) was vortexed thoroughly; then, under adequate lighting, the tube was positioned side by side with the 0.5McFarland standard against a white card

containing several horizontal black lines. The turbidities were compared by looking at the black lines through the suspensions (Fig: 2). After standardization, the inoculum suspensions were used within 15 minutes of preparation.

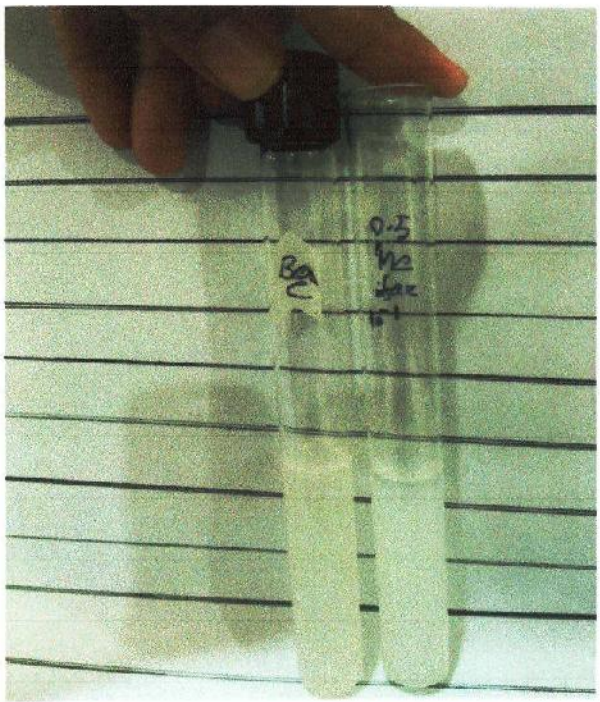

Fig 2: The tube on the right is a McFarland 0.5 turbidity standard. The tube on the left is a test bacterial suspension that has turbidity same that of the McFarland standard;

3.9.2 Disc diffusion test

A McFarland 0.5 standardized suspensions of bacteria (total bacterial and *Vibrio*) was swabbed over the surface of a Nutrient agar plate, and paper discs (4mm diameter) containing specific concentrations (7.5 ppm,15 ppm, 30 ppm, 50 and 60 ppm) of active chlorine were placed onto the inoculated surface. After overnight incubation, the diameters of the zones produced by antimicrobial inhibition of bacterial growth were measured and the isolate was interpreted as either non-susceptible, susceptible, intermediate, or resistant to bleaching powder according to preset criteria. Not only the bleaching powder but also five commercial antibiotic discs named- ampicillin (AMP 10), kanamycin (K 30), novobiocin (NV 30), streptomycin (S 10) and chloramphenicol (C 30) were tested. Oxytetracycline (OTC) capsule

(250 mg) and ciprofloxacin (CP) tablet (250 mg) were prepared as disc and used. These discs were placed aseptically onto Nutrient agar plates with 2% NaCl previously swabbed with the test bacteria (*Vibrio* and total bacteria). After incubating the agar plates for 24 h at 37 °C, the diameter of the inhibition zone observed around each disc was measured and compared to the standard table for antimicrobial susceptibility provided by NCCLS to determine the susceptibility of each isolate to the antibiotics. Additionally, oxytetracycline and ciprofloxacin were used at different concentrations. The two antibiotics were used with concentrations of 5, 7.5, 10, and 12.5 ppm.

3.9.3.1 Determination of Minimum Inhibitory Concentration (MIC)

Broth-Macrodilution (Tube-Dilution) Tests were performed to determine the MIC. Nutrient broth with inocula was prepared from McFarland 0.5 standards (by 1:100 diluton) to obtain a final bacterial concentration of about 5×10^5 CFU/ml. 2 ml of broth with inocula were taken in test tubes. Then 2 ml of antimicrobial agent (100 µg/ml=100 ppm of active chlorine), was added to the 1st test tube. By fourfold serial dilution the concentration of the active chlorine was decreased to 50 µg/ml, 30 µg/ml, 15 µg/ml, 7.5 µg/ml. A concentration of 60 ppm was used also. A growth-control tube (broth plus inoculum) and an uninoculated control tube (broth only) were used with each test. After overnight incubation at 35° C, the MIC was determined by visually as the lowest concentration that inhibits growth, as demonstrated by the absence of turbidity. The MIC of oxytetracycline and ciprofloxacin was determined at different concentrations by visually as the lowest concentration that inhibits growth. Concentrations of 5, 7.5, 10 and 12.5 ppm for oxytetracycline and ciprofloxacin were used.

3.9.3.2 Spectrophotometer test

MIC of bleaching powder was determined also by using Spectrophotometer. Nutrient broth with inocula was prepared from McFarland 0.5 standards (by 1:100 diluton) to obtain a final bacterial concentration of about 5×10^5 CFU/ml. 2 ml of broth with inocula were taken in test tubes. Then 2 ml of antimicrobial agent (100 ppm of active chlorine), was added to the 1st test tube. By fourfold serial dilution the concentration of the active chlorine was decreased to 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25µg/ml. A concentration of 15 ppm was used also. A growth-control tube (broth plus inoculum) and an uninoculated control tube (broth only) were used with each test. After overnight incubation at 35° C, the MIC was determined by spectrophotometer (HITACHI, U-2910) by measuring optical density (OD) at 600 nm(nano meter).

3.9.4 Determination of Minimum Bactericidal Concentration (MBC)

After MIC determination of the active chlorine tested, an aliquot of 100 µl from all tubes in which no visible bacterial growth was observed were seeded in Nutrient Agar (NA) plates not supplemented with active chlorine. The plates were then incubated for overnight at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the NA plates. MBC was determined according to Petrus, E. M., *et al.* (2011).

CHAPTER FOUR

RESULTS

4. Results

The study was conducted to observe the effect of bleaching powder ($Ca(ClO)_2$) solution on total bacterial load and *Vibrio* spp and to determine the optimum doses of bleaching powder as well as to observed antibiotic susceptibility patterns of total bacteria and *Vibrio* isolates. The study was conducted at the Biochemistry and Molecular Genetics Lab of Khulna University.

4.1 Bacterial identification

Total bacterial load and *Vibrio* spp. were isolated from the prawn larvae and treated water which were collected from the hatchery.Table1 showed the bacterial load obtained from larvae and treated water (from larval rearing tanks)sample .

Table1. Bacterial load from hatchery water and larvae.

Source	**Bacterial load (cfu/ml)**		**Source**	**Bacterial load(cfu/g)**	
Water	Total bacterial bacterial load	*Vibrio* bacterial load	**Prawn larvae**	Total bacterial bacterial load	*Vibrio* bacterial load
	5.8×10^2	**3.3×10^2**		**9.3×10^2**	**5.5×10^2**

Most of the bacteria were *Vibrio* among the total bacteria those were isolated. After completing the biochemical tests two types of bacterial (*Vibrio*) spp were identified. These were *Vibrio harveyi* (71.5%) and *Vibrio parahaemolyticus* (28.5%). Then the pure isolates were preserved in Nutrient Broth (HIMEDIA) with 2% NaCl, in 30% glycerol and stored at -80 °C until use.

4.2 Determination of active chlorine

Three different available commercial bleaching powders were tested to determine the percentage of available active chlorine. Table2 showed the amount of active chlorine that was measured.

Table2. Percentage of available active chlorine.

Name of bleaching powder brands	% active chlorine written in the packet	% active chlorine found
HI (Japan)	65	53
KCI (India)	35	28
Local	25	12

4.3 Antimicrobial susceptibility test

4.3.1 Disk Diffusion Test

Paper disks (4mm diameter) containing specific concentrations of antimicrobial agent active chlorine were placed onto the inoculated surface. After overnight incubation, the diameters of the zones of inhibition produced by bleaching powder solution were measured. Different concentrations produced Different diameters of the zones of inhibition (table 3).

Table3. Mean diameter of zones of inhibition (mm) for active chlorine at varying concentrations in disk diffusion test.

Concentrations (µg/ml=ppm)		Zones of inhibition (mm)	
	Total bacteria	*Vibrio harveyi*	*Vibrio parahaemolyticus*
7.5	Negligible	No	No
15	*15*	No	Negligible
30	23	13	13
50	Too large	*19*	*19*
60	Too large	20	21

Approximately a clear zone of inhibition for total bacteria was found in the concentrations of 15 ppm active chlorine. On the other hand, in the concentrations of 50 ppm of active chlorine against *Vibrio harveyi* and *Vibrio parahaemolyticus* produced clear zones of inhibition. The different zones of inhibition produced by bleaching powder solution were presented in Fig: 3.

Five commercial antibiotic discs named- Ampicillin (AMP 10), Kanamycin (K 30), Novobiocin (NV 30), Streptomycin (S 10) and Chloramphenicol (C 30) were tested to observe the zones of inhibition. Additionally oxytetracycline (OTC) and ciprofloxacin (CP) were prepared as disc to observe the zones of inhibition (table4).

Table4. Mean diameter of zones of inhibition (mm) for various antibiotics.

Antibiotics	Disc content (µg)	Zones of inhibition (mm)		
		Total bacteria	*Vibrio harveyi*	*Vibrio parahaemolyticus*
Ampicillin (AMP)	10	10	No	No
Chloramphenicol (C)	30	26	21	20
Kanamycin (K)	30	22	17	17
Novobiocin (NV)	30	17	14	15
Streptomycin (S)	10	9	No	No
Oxytetracycline (OTC)	30	20	14	15
Ciprofloxacin (CP)	10	19	15	15

Ampicillin against total bacteria produced a zone of 10 mm and produced 9 mm zone by streptomycin. Among these antibiotics chloramphenicol (30) showed moderately good clear zone for *Vibrio*. But in the case of kanamycin (30) and novobiocin (30) showed the zones of inhibition that contained few bacteria scattered on the zone. Oxytetracycline (30) and ciprofloxacin (10) didn't produce good clear zone for *Vibrio*. Streptomycin (10) and ampicillin (10) produced no zones of inhibition against *Vibrio*. Different zones of inhibition produced by antibiotics were presented in Fig: 4.

Table6. Optical density at different concentrations of active chlorine against sample bacteria

Concentrations of active chlorine(ppm)	Optical density (OD) at 600 nm	
	Total bacteria	*Vibrio spp*
6.25	0.305	0.923
12.5	0.154	0.502
15	*0.125*	0.431
25	0.075	0.249
50	0.001	*0.122*

Growth-control tube (broth plus inoculum) showed the Optical density of 0.925 that was prepared from McFarland 0.5 standards (by 1:100 diluton). The lowest optical density was measured at the concentration of 15 ppm for total bacteria and 50 ppm for *Vibrio spp.*

4.3.3 Minimum Bactericidal Concentration (MBC)

After MIC determination of the active chlorine tested, an aliquot of 100 µl from all tubes in which no visible bacterial growth was observed were seeded in Nutrient Agar (NA) plates not supplemented with active chlorine. The plates were then incubated for overnight at 37°C. The MBC value of active chlorine for total bacteria was 17.5 ppm. And MBC values of active chlorine against *Vibrio harveyi* and *Vibrio parahaemolyticus* were approximately same as their MIC values. The value was 52.5 ppm.These concentrations absolutely killed the bacteria.

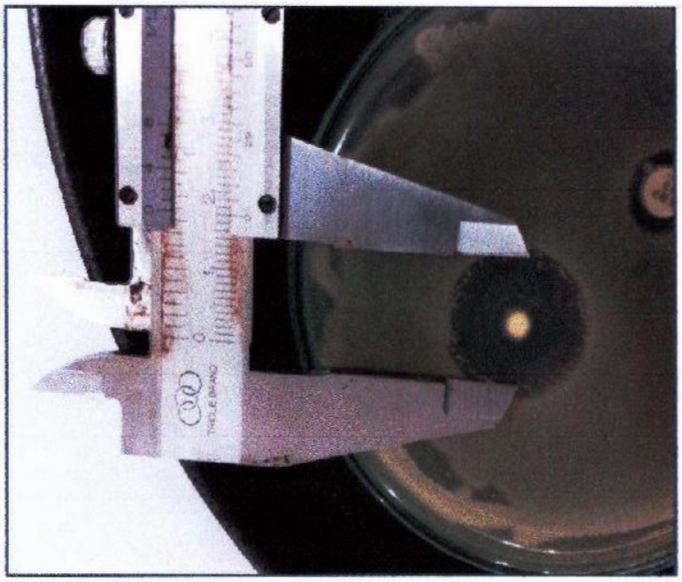

Fig 3 (a): Zone of inhibition produced by disc contained active chlorine (15 ppm) against total bacteria was examined by slide calipers

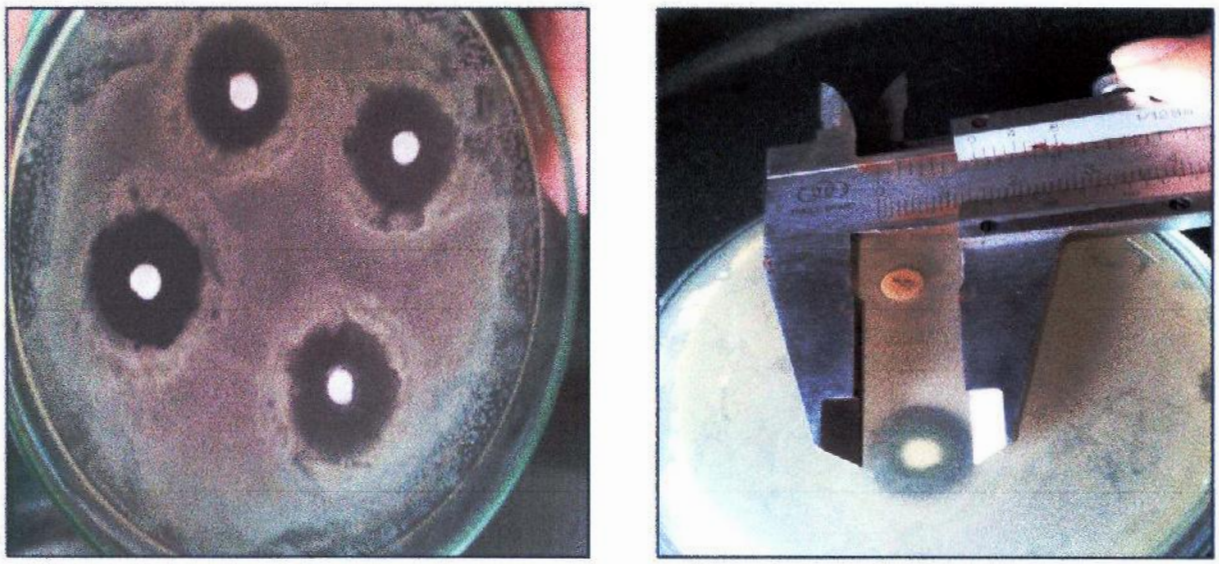

Fig 3 (b): Zone of inhibition produced by disc contained active chlorine (50ppm) against *Vibrio* was examined by slide calipers

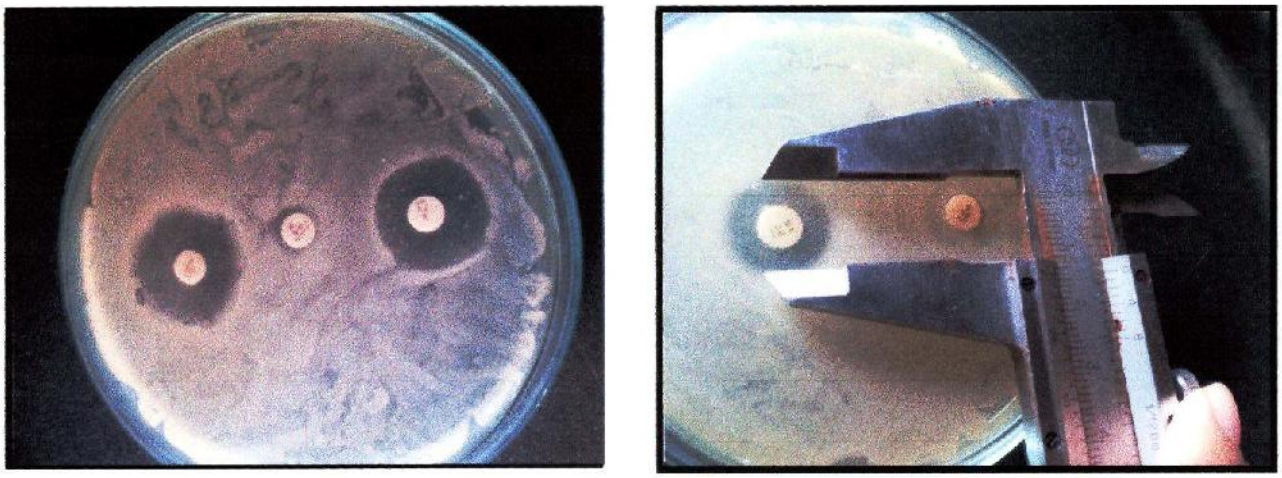

Fig 4 (a): Zone of inhibition produced by antibiotics (from left to right) chloramphenicol, ampicillin, kanamycin, novobiocin and streptomycin discs against *Vibrio* spp

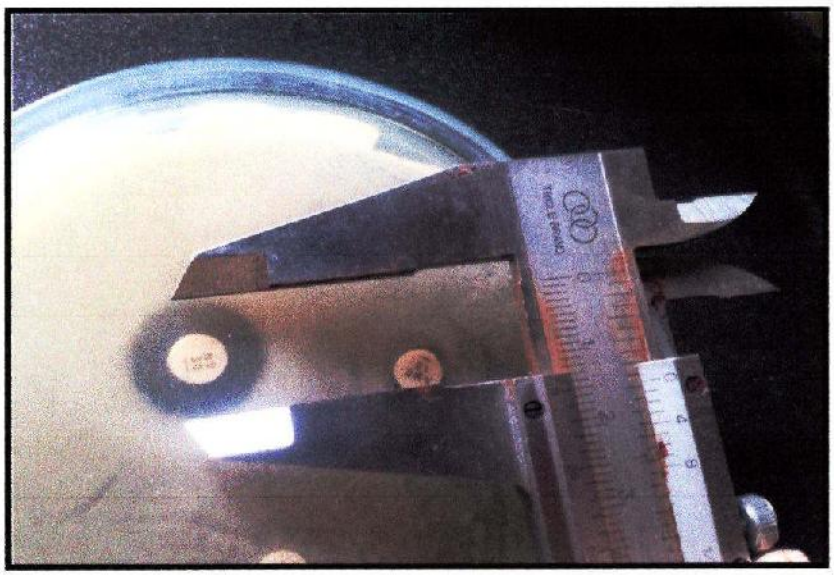

Fig 4(b): Zone of inhibition produced by Novobiocin disc against *Vibrio* was examined by slide calipers

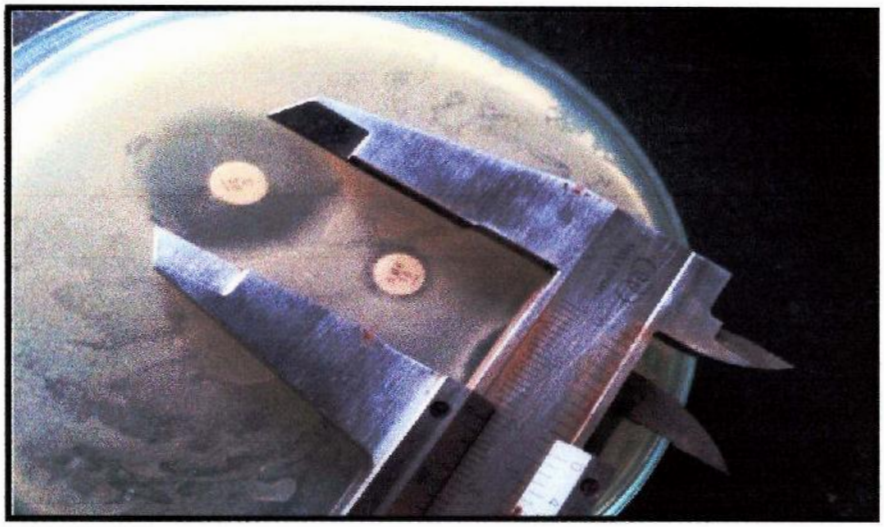

Fig 4(c): Zone of inhibition produced by Chloramphenicol disc against *Vibrio* was examined by slide calipers

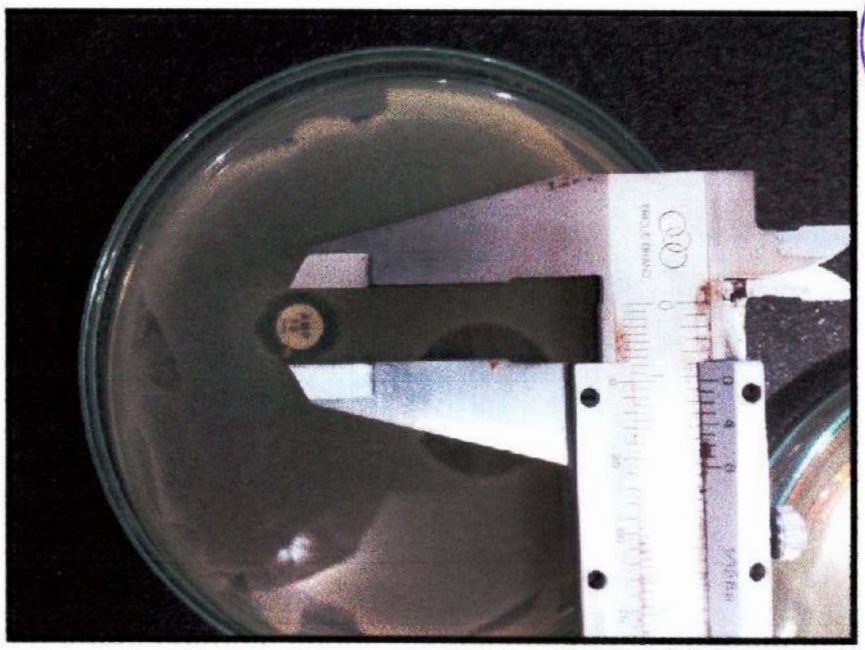

Fig 4 (d): Zone of inhibition produced by ampicillin disc against total bacteria was examined by slide calipers

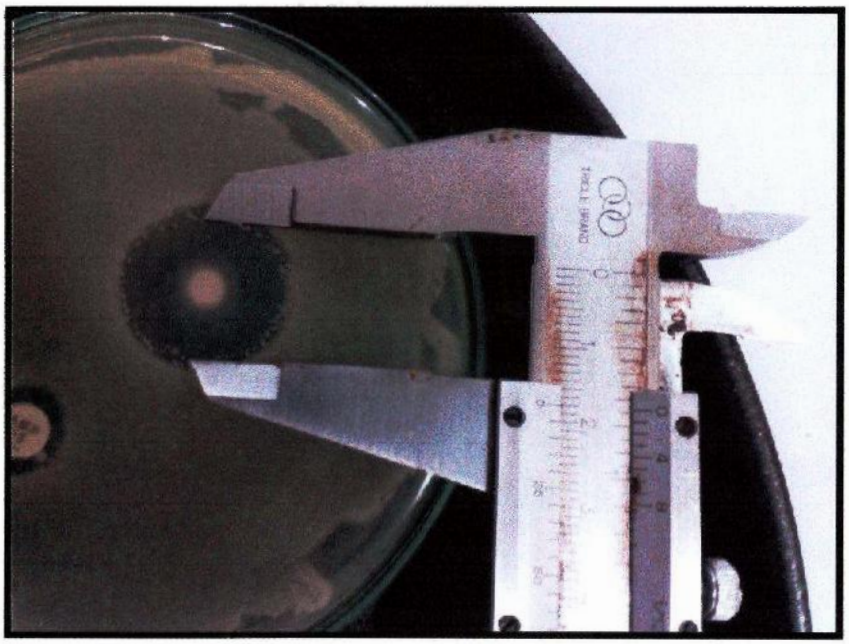

Fig 5(a): Zone of inhibition produced by disc contained Oxytetracycline (12.5 ppm) against *Vibrio* was examined by slide calipers

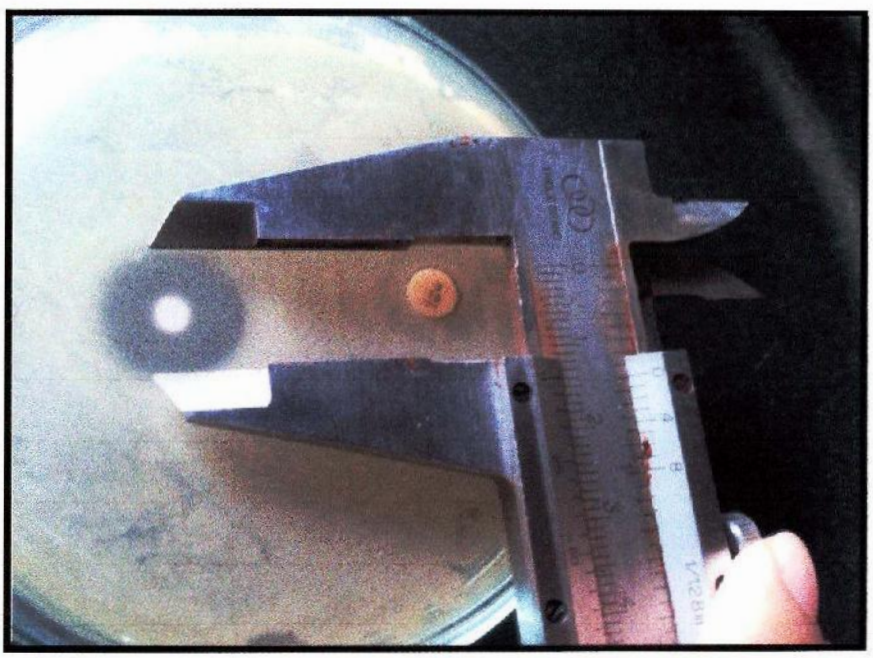

Fig 5(b): Zone of inhibition produced by disc contained Ciprofloxacin (10 ppm) against *Vibrio* was examined by slide calipers

CHAPTER FIVE

DISCUSSION

5. Discussion

The hatchery business for prawns (*Macrobrachium rosenbergii*, known locally as golda) is relatively new in Bangladesh and has increased over the past few years due to an increasing demand for hatchery reared post larvae (PL). It is known from Abedin *et al.*, (2001), The construction of freshwater ghers has increased by 10-20 percent per annum, and is now the primary livelihood strategy of more than 100,000 rural households in the region (Biswas and Finan, 2004). But mass mortalities of larvae and post larvae in prawn hatcheries consecutively for the last few years have been turning many prawn hatcheries into total bankrupt. De la Pena *et al.*, (2001), observed that diseases caused by members of the genus *Vibrio* are often the limiting factor in shrimp and prawn culture operations worldwide. In the present study, the two main species of *Vibrio* isolated were *Vibrio harveyi* and *Vibrio parahaemolyticus* comprising 71.5% and 28.5% of collected prawn larvae and water sample. Luminescent *Vibrio harveyi* appears to release exotoxins (Liu *et al.*, 1996) and may cause 80-100% mortality in *Penaeus monodon* hatcheries. *Vibrio harveyi* and *Vibrio parahaemolyticus* are associated with the diseased shrimp. There is recent evidence to suggest that *Vibrio harveyi* can survive in pond sediment even after chlorination or treatment with lime (Karunasagar *et al.*, 1996). Larval prawns are particularly susceptible to *Vibrio harvey* (Lavilla-Pitogo *et al.*, 1990). The present study also observed the presence of *Vibrio harvey* in Larval prawns. It is thus imperative that the pathogens especially *Vibrio* spp. in prawn hatchery system must be controlled for a successful hatchery operation.

Bleaching powder is used in most prawn hatcheries in the form of calcium hypochlorite ($Ca(ClO)_2$) for water disinfection. Active chlorine present in bleaching powder that inhibits the bacterial growth and/or kills the bacteria, not the bleaching powder itself. But the percentage of active chlorine is not correctly written in the packets of bleaching powder. In the present study it was observed. In the present study it was found that total bacteria in prawn hatchery were not inhibited by using optimum doses of bleaching powder that were usually recommended (12-15 ppm). It was needed (30-35) ppm bleaching powder that contained approximately 50% active chlorine, which means 15 ppm of active chlorine was needed to inhibit the total bacterial growth in prawn hatchery. In this study the MIC of active chlorine on total bacteria was 15 ppm. From this study it was found that increasing doses of active chlorine can inhibit more bacterial growth. In the case of 15 ppm dose, it was unable to produce any zone of inhibition against *Vibrio harvey*. A zone of inhibition was produced (but

not clear) by doubling the dose (30 ppm). In the case of *Vibrio harvey* and *Vibrio parahaemolyticus* the optimum dose of active chlorine was 50 ppm in the study, but it is impractical. So it can be said from the present study that, bleaching powder is unable to kill the pathogenic *Vibrio* spp, even it is unable to inhibit them.

Being the high doses of bleaching powder, different types of antibiotics were also used in the study. Kanamycin (30) didn't produce clear zone of inhibition against Vibrio harveyi and *Vibrio parahaemolyticus*. Similarly, Baticados *et al.* (1990) also reported *Vibrio harveyi* showed resistance to kanamycin, penicillin G, and streptomycin. Novobiocin (30) showed the zones of inhibition that contained few bacteria scattered on the zone. Streptomycin (10) and ampicillin (10) produced no zones of inhibition for *Vibrio harvey* and *Vibrio parahaemolyticus*. That means all the *Vibrio* isolates were found to be resistant to ampicillin and Streptomycin. Similarly, Manjusha *et al.*, (2005) also reported that environmental *Vibrio* isolates were the most resistant to ampicillin. In the present study only chloramphenicol (30) showed sensivity against both the total bacteria and *Vibrio*. Similar report was found by Ransangan, J. *et al.*, (2013). Although, in this present study Chloramphenicol (30) produced clear zone of inhibition but, its use is not encouraged as there are concerns regarding its embryo- and fetotoxicity, its carcinogenic potential and its potential for causing a dose-dependent aplastic anemia in humans 3 to 12 months after exposure (WHO 2006). United States of America Federal regulations prohibit its use in food-producing animals and animal-feed products (FDA, 2002b). Oxytetracycline and ciprofloxacin are used in most of the hatchery in the locality at a dose of 2-3 ppm (Dr. Matthew Briggs, 2013). But, in the study these antibiotics were found unable to inhibit the pathogenic *Vibrio* spp at that dose.

Oxytetracycline and Ciprofloxacin can be used in higher doses. The MIC value of oxytetracycline was 12.5 ppm and ciprofloxacin was 10 ppm against *Vibrio* spp. In the present study it was obtained that, MIC for both *Vibrio harveyi* and *Vibrio parahaemolyticus* was approximately same. So it can be said that, the two pathogens will be treated with similar doses of antibacterial agents. But the high doses are not recommended because, it has environmental hazard and as well as economical hazard.

Vibrio harveyi is a ubiquitous species, the bacterial load of this species should be carefully observed since it is also a known agent of vibriosis. Austin and Austin (1993) reported that *Vibrio* species occur widely in aquatic environments and part of the normal flora of coastal

seawater. They also exist as normal flora in fish and shellfish but have also been recognized as opportunistic pathagens in many marine animals. As two *Vibrio* species was found in the present study associated with larval prawn, use of optimized doses of active chlorine will be helpful for the hatchery operation, but the optimized doses of active chlorine were too high and most of antibiotics were resistant to *Vibrio harveyi*, so further study about strain level is recommended. Compared to agricultural use and medicinal use the market for aquaculture antimicrobials is fairly small and the approval process can be expensive. In fact, if we take the USA as an example, only five drugs have been approved by the US Food and Drug Administration (FDA) for disease-treatment (Schnick, 2000). The accumulated scientific evidence is that certain uses of antibiotics in food producing animals can lead to antibiotic resistance in intestinal bacteria, and this resistance can then be transmitted to the general population, causing treatment-resistant illness. These uses of antibiotics can also create antibiotic resistance in non-pathogenic bacteria, the resistance genes of which can be transferred to disease-causing bacteria, resulting in antibiotic-resistant infections for humans. So extreme care should be taken in the selection of antimicrobial agents to control pathogenic *Vibrio* spp. All drugs legally used in aquaculture must be approved by the government agency responsible for veterinary medicine, for example, the Food and Drug Administration (FDA) in the USA (Romero, J. *et al*). Fish do not effectively metabolize antibiotics and will pass them largely unused back into the environment in feces. It has been estimated that 75 percent of the antibiotics fed to fish are excreted into the water (Burridge *et al.*, 2010). Samuelsen, (2006); Ellingsen *et al.*, (2002) reported that, Oxytetracycline (OTC), one of the most commonly used antibiotics in fish farms and hatcheries, is very poorly absorbed through the intestinal tract of fish. It has been estimated that 70-80% of OTC is intact in the feces. So antibiotics should be used with proper way that can minimize environmental pollution. Water should be treated before excretion into the natural water.

The rise in bacterial antibiotic resistance and antibiotic residues has become global concerns, and there is a need to develop alternative therapies for bacterial pathogens in animal production, especially in aquaculture. Vaccination is an ideal method for preventing infectious diseases, but it is not a treatment for existing infections, and commercially available vaccines are still very limited in the aquaculture field. Several alternatives to the use of antibiotics have been used successfully in aquaculture. The use of innocuous microorganisms to avoid bacterial infection in aquatic organisms has been tested in

aquaculture. Use of probiotics can be fruitful for prawn hatchery operation (Romero, J. *et al.*2012).

Another source of alternative treatments is essential oils, which are natural components from plants that are generally recognized as safe substances (GRAS). Due to their antimicrobial properties, these oils may constitute alternative prophylactic and therapeutic agents in aquaculture (Romero, J. *et al.* 2012).

Proper hatchery management can minimize most of bacterial hazards. The major implementation of any biosecurity measures is always going to be in the hatcheries and in the growing ponds. However, besides good management practices and treatments in hatcheries and ponds there are biosecurity measures which should be put into place (Aquaculture – Biosecurity, http://www.google.com):

All vectors that can transmit disease from one place to another should be identified. Man is a major source of contamination. Anybody working with shrimp in several ponds should wash his/her hands, legs and feet with proper disinfection solutions, after handling equipment or animals and before moving to work on the next pond. Other vectors of disease transmission include crabs, rodents wild birds etc. which can contaminate the water in ponds. These vectors should be kept in control completely in the vicinity of aquaculture practices. Equipment should not share between ponds unless necessary. In these cases, all equipments should be disinfected prior to being re-used. All surrounding areas of the hatchery or farm should be kept clean. Water treatments of incoming or recirculating water decreases the chance of pathogenic organisms entering the culture system. Treatments include mechanical filtration, UV light, and ozone. Treatment of wastewater from aquaculture facilities and processing plants reduces the release of microorganisms into the environment. This is important, because there have been problems in the past with diseases re-entering an aquaculture facility from wastewater of a processing plant. Infected water from the plant was released, and then this water was taken into the aquaculture system. It is important to use clean, fresh feed. Proper handling and storage of feed can reduce food-borne disease organisms. The proper disposal of mortalities by incineration, burial, or composting will reduce the risk of recycling diseases. It is also important to remove dead fish from the tank or raceway often to reduce the likelihood of spreading the infection.

Lightner (2003) discussed ways of excluding pathogens from stock (i.e., post larvae and broodstock), especially through the use of quarantine and specific pathogen-free (SPF) certified stocks, and restricting imports of live and frozen shrimp. Excluding vectors and external sources of contamination and preventing internal cross contamination were suggested methods for excluding pathogens from hatcheries and farms. Horowitz and Horowitz (2003) suggested providing better environmental and biological conditions to the infected population to increase its ability to resist diseases. They discussed the following steps: a) effect physical measures (increase aeration, control temperature, improve the feeding regime, remove sludge and organic matter, and treat wastewater) to improve the environmental conditions, b) effect chemical measures, including control of PH and salinity, reduction of ammonia and nitrite, and application of antibiotics, and c) to use effective biological measures, consisting mainly of the use of probiotics containing a mix of bacterial species to establish beneficial microbial communities under culture conditions.

From the above discussion it can be said that by maintaining all these things, the present problem of prawn hatcheries can be minimized.

However, there were some limitations in the study. Active chlorine was decreased day by day. So it is recommended that bleaching powder must be used immediately after opening the packet. And it is also crucial to do further study about this vibriosis problem.

CHAPTER SIX

CONCLUSION

6. Conclusion

Bangladesh is blessed with giant river prawn (*Macrobrachium rosenbergii*, locally known as golda) farming because of its favourable resources, including vast inland freshwater and adjacent brackishwater areas, geographic location and agro climatic conditions. Geographically Bangladesh enjoys a series of natural advantages for prawn culture. Its soil, water, climate and local cultural tradition are congenial for giant river prawn (also known as freshwater prawn) production. It is recognized that prawn farming is an important aquaculture activity in Bangladesh and plays significant role in poverty alleviation, employment generation, foreign currency earning and improvement of socioeconomic conditions of farmers and associated groups. But in recent years prawn farming are in great threat. Prawn PL cannot be produced perfectly. The main reason is still unknown. However, mortality due to pathogenic *Vibrio* spp may one of the major causes.

In this present study it was observed that one of the major causes of mortality of prawn PL is pathogenic *Vibrio* spp. As percentage of active chlorine are found less than that written on the packet of bleaching powder so, bleaching powder should be used by measuring active chlorine. Some antibiotics can be used at optimum doses. However, further studies that dealing with genetical analysis of the prawn PL as well as analysis of *Vibrio* spp at strain level are necessary to conclusively establish the main cause of the drastic mortality of prawn PL.

CHAPTER SEVEN

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PHOTO GALLERY

Photo Gallery

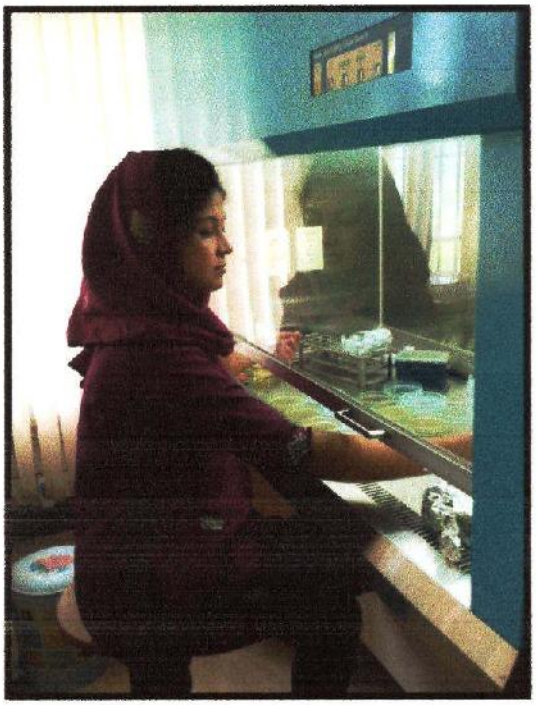

Fig: Total plate count

Fig: Citrate test

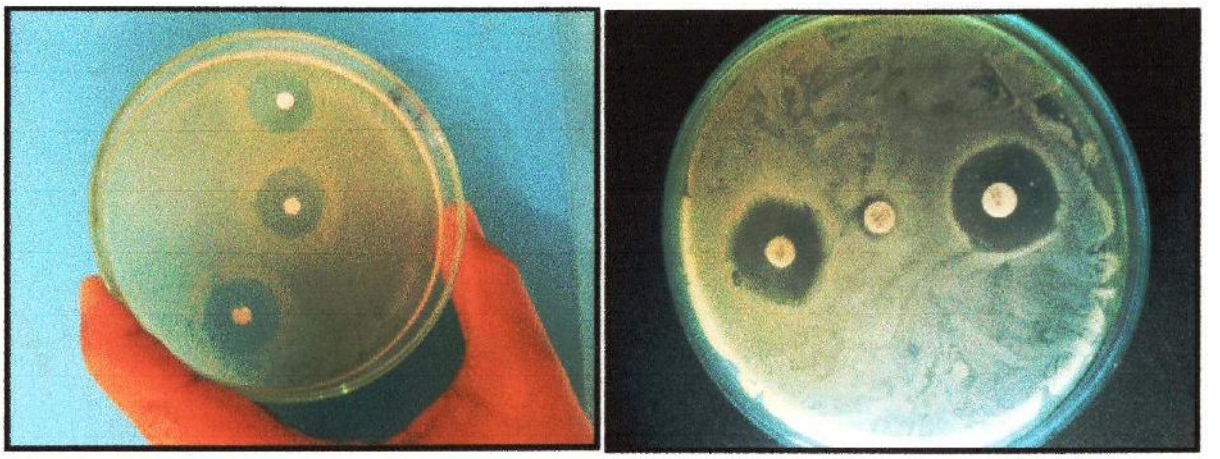

Fig: Zone of inhibition produced by bleaching solution (left) and antibiotics (right).