BACTERIAL LOAD AND 16S rRNA SEQUENCE BASED IDENTIFICATION OF *VIBRIO* spp. IN FISH AND SHRIMP HATCHERIES OF BANGLADESH

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We certify that the research work embodied in this thesis entitled "**Bacterial Load and 16S rRNA Sequence Based Identification of *Vibrio* spp. in Fish and Shrimp Hatcheries of Bangladesh**" submitted by **Md. Abdur Razzak Hasan**, Roll No.: Curzon-704, Session: 2014-15, Registration No.: 2010-312-993/2010-11, has been carried out under our supervision.

This is further to certify that it is an original work and suitable for the partial fulfillment of the degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

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The Author

Abstract

The presence of *Vibrio* spp., one of the deadliest fish and shrimp pathogen in aquaculture facilities worldwide for which hatchery owners often suffer hectic economic losses, were observed in this study with species level identification in three shrimp and fish hatcheries of Cox's Bazar, Mymensingh and Bogra, Bangladesh.

Bacterial enumeration was done in nutrient agar (NA), marine agar (MA) and thiosulphate citrate bile salt sucrose agar (TCBS) plate to understand the microbial load in the corresponding shrimp, tilapia, shing, magur and pangas fry rearing environment which will provide an insight into the environmental management implications and need for further initiatives.

Artemia hatching tank of Zomzom hatchery, Cox's Bazar had similar total bacterial build up ($2.59 \pm 0.10\times10^7$ cfu/g) in the water sampled and in the shrimp post larvae (PL) sampled at stage 10 and 12 ($2.37 \pm 0.11\times10^7$ cfu/g and $2.42 \pm 0.10\times10^7$ cfu/g respectively). However, bacterial load determined from the samples of water corresponding to the stages of PL were similar but different from the samples of *Artemia* tank and PL stages of 10 and 12. In MA plate, no significant differences were observed in the bacterial count of these samples. Similar result was observed for the total presumptive vibrio count in TCBS plates which ranged from $3.8 \pm 0.60\times10^3$ cfu/g to $1.62 \pm 0.50\times10^3$ cfu/g.

Total bacterial load ($7.5 \pm 0.11\times10^7$) measured in the water sampled from 25 day old fry rearing pond of tilapia, from Reliance Tilapia Hatchery, Mymensingh, was similar to that of 33 day old fry ($8.6 \pm .66\times10^7$). The bacterial density found in the 25 ($1.6 \pm 0.50\times10^7$), 28 ($3.12 \pm 0.14\times10^7$) and 40 day old fry ($6.46 \pm 1.52\times10^6$) samples were similar but significantly different from the sample of 33 day old fry and the water sample of the pond of 25 day old fry. In TCBS plate, bacterial abundance detected in the samples across all four age groups were similar (25 day old fry: $4.21 \pm 3.79\times10^3$; 28 day old fry: $4.90 \pm 3.50\times10^3$; 33 day old fry: $1.08 \pm 0.12\times10^3$; 40 day old fry: $7.04 \pm 2.08\times10^3$). No bacterial count was found in the water sampled from 25 day old fry rearing pond.

In GM Aquaculture Ltd, Bogra, the overall bacterial build up ($2.03 \pm 0.31\times10^8$) found in the samples of zeol fish fry in NA plate was significantly higher than that of thecorresponding rearing pond water ($2.11 \pm 0.459\times10^7$) and the water of the live food rearing tank ($8.43 \pm 0.57\times10^6$). Similar to that, TCBS plates had 2.3-, and 5.09-folds higher bacterial load ($1.08\pm 0.25\times10^3$) in the samples of fish fry than in the samples of the corresponding water samples and water samples of the live food rearing tank, respectively ($4.70 \pm 1.67\times10^2$ and $2.12 \pm 0.28\times10^2$).

37 *Vibrio* colonies, selected based on their morphological dissimilarities in TCBS plate, were subjected to amplified 16S ribosomal DNA restriction analysis (ARDRA) using *Alu*I restriction enzymefollowing their DNA extraction and amplification of 16S rRNA (1450 bp). From this analysis, ultimately 8 groups (representative isolates), named as ARH 1, ARH 2, ARH 3, ARH 4, ARH 5, ARH 6, ARH 7 and ARH 8, of different band pattern were sequenced and identified as *Vibrio alginolyticus*, *Aeromonas veronii, Aeromonas hydrophila, Vibrio vulnificus, Vibrio cholera, Edwardsiella hoshinae, Bacillus methylotrophicus* and *Aeromonas veronii*,respectively.

Polymorphic sites among the sequenced strains were studied by multiple sequence alignment considering 1320 bp nucleotides where 12.9% dissimilarities were observed among the identified *Aeromonas* and *Vibrio* species which is 5.7% among 3 *Vibrio* species. Phylogenetic analysis also confirmed the taxonomic relation among the identified species.

Vibrio species identified in this study, are pathogenic for human and aquatic organisms, and were found only in shrimp hatchery with the dominance of *V. alginolyticus*. Findings of this study indicate the poor quality of water treatment and management of the hatchery. It is also observed that all these 3 *Vibrio* species were present in the *Artemia* rearing tank which also indicates the possible source of pathogens.

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List of Symbols and Abbreviations

Symbols	**Details**
°C	Degree Celsius
µg	Microgram
µL	Microliter
AAI	average amino acid identity
APHA	American Public Health Association
APW	Alkaline Peptone Water
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATCC	American Type Culture Collection
bp	Base pair
CFU	Colony Forming Unit
cm	Centimeter
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytidine triphosphate
dGTP	Deoxy guanidine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxy thymidine triphosphate
EDTA	Ethylenediamine tetra acetic acid
eg	For example (exempli gratia)
et al	And others (et alliori)
EtBr	Ethidium Bromide
LB	Luria-Bertani
MA	Marine Agar
MCT	Micro Centrifuge Tube
MEGA	Molecular Evolutionary Genetics Analysis
mL	Milliliter
mm	Millimeter
NA	Nutrient Agar
NaCl	Sodium Chloride
ng	Nanogram
PCR	Polymerase chain reaction
PL	Post Larvae
Symbols	**Details**

RNase	Ribonuclease
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic Acid
spp	Species
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TBC	Total Bacterial Colony
TBE	Tris borate EDTA
TCBS	Thiosulfate Citrate Bile Salt Sucrose agar
TE	Tris-EDTA
V.	*Vibrio*
g	Gram
μm	Micrometer
MLSA	Multi Locus Sequence Analysis
mM	millimolar
aw	water activity
DDH	DNA-DNA hybridization
v/v	Volume per volume
w/v	Weight per volume
MP	Maximum parsimony
ML	Maximum likelihood
NJ	Neighbor- joining
STEC	Shiga toxin–producing *Escherichia coli*
CDC	Centers for Disease Control and Prevention
MMWR	Morbidity and Mortality Weekly Report
ANOVA	Analysis of Variance
SEM	Standard Error of Mean
HSD	Honestly Significant Difference
EMS	Early Mortality Syndrome

Chapter 1

Introduction

1.1 Background

Vibrio is one of the six genera under the family Vibrionaceae. *Vibrio* species is defined as a group of strains forming small (0.5–0.8 1.4–2.6 µm) rods with polar flagella enclosed in a sheath, facultative anaerobic metabolism, capable of fermenting D-glucose and growth at 20°C (Gomez-Gil *et al.,* 2014). To date, one hundred and thirty species of *Vibrios* have been described and twelve were classified as human pathogens implicated mostly in food- or water-borne diseases (Thompson *et al.,* 2006), including *V. cholerae* as the main cause of diarrhea, *V. parahaemolyticus* as the cause of foodborne gastroenteritis (Ozer *et al*., 2008; Pruzzo *et al*., 2005) and *V. vulnificus* which is known to cause 95% of all deaths associated with seafood consumption (Rosche *et al*., 2006). Food safety is a crucial concern (Jacxsens *et al*., 2009) and marine products as the main source of a large number of pathogenic bacteria, including *Vibrio* spp., need to be taken more into consideration.

Vibrios occur in a wide range of aquatic environments found, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Barbieri *et al.,* 1999; Urakawa *et al.,* 2000; Suantika *et al.,* 2001; Thompson *et al.,* 2001; Heidelberg *et al.,* 2002; Vandenberghe *et al.,* 2003; Venter *et al.,* 2004). Several cultivation-dependent and independent studies have shown that Vibrios appear particularly in high densities in and/or on marine organisms, e.g., corals (Rosenberg and Ben Haim 2002), fish (Huys *et al.,* 2001), gorgonians (Martin *et al.,* 2002), shellfish (Sawabe *et al.,* 2003), sea grass (Weidner *et al.,* 2000), sponges (Hentschel *et al.,* 2001), shrimps (Gomez-Gil *et al.,* 1998), squids (Ruby 1996; Nishiguchi, 2000), and zooplankton (Heidelberg *et al.,* 2002). Halophilic Vibrios can represent as much as 40% of the total microbiota of subtropical coastal water (Chan *et al.,* 1986).

Mostly they are tolerant to alkaline pH, but sensitive to acid pH. Because of high content of sodium chloride in the habitat, they can stand lower water activity (aw) which is 0.980 (Madigan *et al*., 2004), but there are also some non-halophilic *Vibrio* species, based on their sodium chloride requirement.

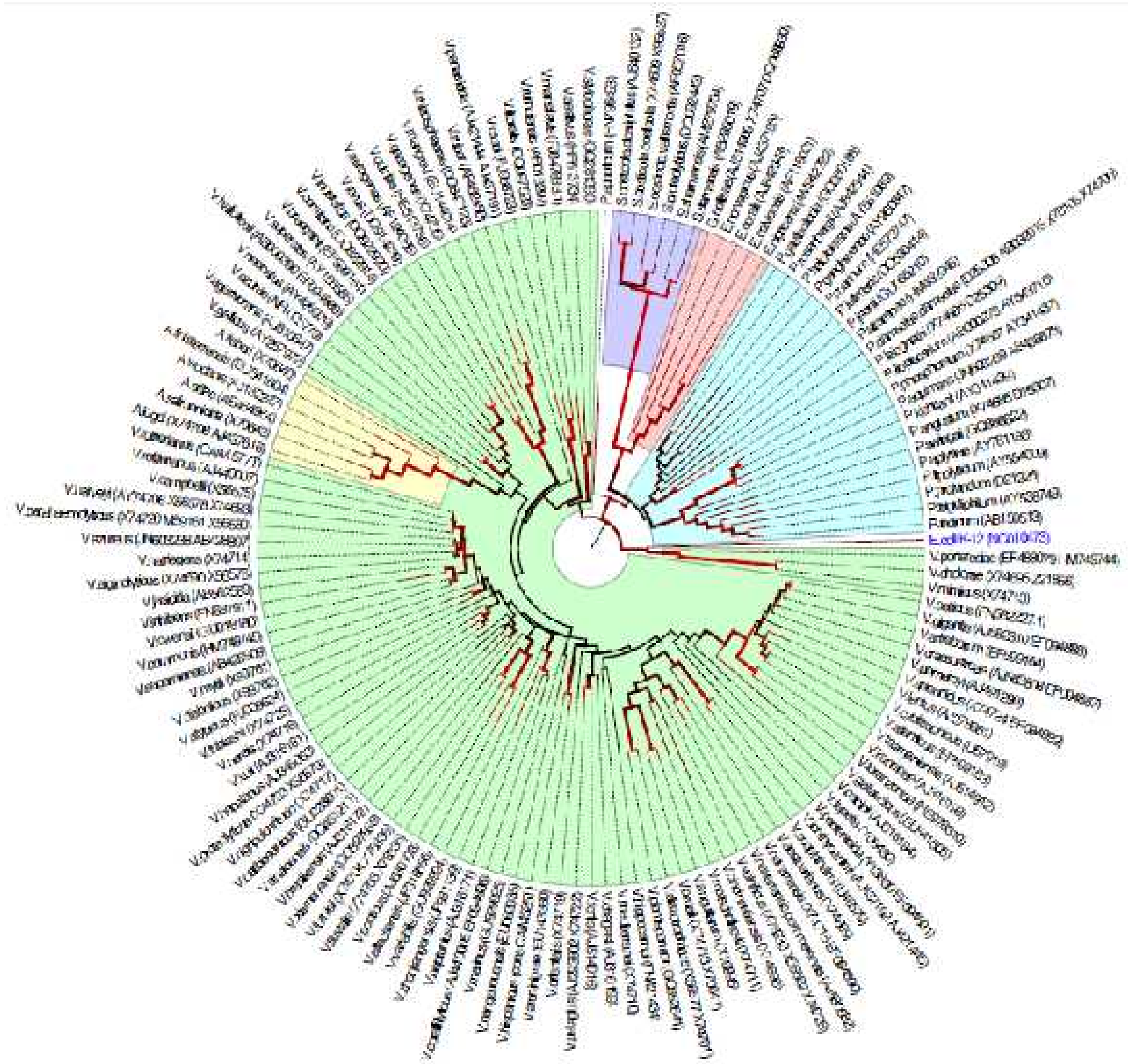


Figure 1.1: 16S rRNA based dendrogram of *Vibrio* species

(Source: Association of *Vibrio* biologists)

Except for the *Vibrio* species that are non-halophilic, such as *V. cholerae* and *V. mimicus*, other *Vibrio* species have the requirement of saline for their growth. Cytochrome oxidase is produced by the majority of *Vibrio* spp. and they show positive result to oxidase test and this can be used as a characteristic in order to differentiate them from the other enteric microorganisms (Enterobacteriaceae) such as *Escherichia coli* (Madigan *et al*., 2004). Besides, *Vibrio* spp. produce catalase, and as a matter of fact in catalase test, they produce observable bubbles.

As mentioned before, *Vibrio* species are facultative anaerobes. And in both aerobic and anaerobic environments they are able to undergo respiratory and fermentative

metabolism, respectively. In addition, they can ferment sugars without producing gas and hydrogen sulphide (Madigan *et al*., 2004). Hence, they can be distinguished from the *Aeromonas* group by their failure in gas production.

Among *Vibrio* spp., there are 12 species which have been proved, by different investigations, to be human pathogens that cause diseases associated with seafood (Janda *et al*., 1988; Holmberg, 1992; Farmer *et al*., 2003; Khaira & *Galanis*, 2007). These *Vibrio* species have been frequently reported as an important cause of gastrointestinal diseases, acute septicaemia and skin infections in humans either by consumption of contaminated seafood or by exposure to aquatic environments (Ottaviani *et al.,* 2009). One of the most important species is *V. cholerae* and particularly serotypes O1 and O139, as the main cause of diarrhea. There are other pathogenic serotypes of *V. cholerae* but they cause less severe diarrhea. *V. parahaemolyticus* has been frequently known as the cause of foodborne gastroenteritis outbreaks in the world (Ozer *et al*., 2008; Pruzzo *et al*., 2005). *V. vulnificus* causes 95% of all deaths associated with the consumption of seafood products (Rosche *et al*., 2006). These three species have been known as the most frequent causes of foodborne illnesses. Other pathogenic species includes *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii,* and*V. mimicus* (Pruzzo *et al*., 2005).

Vibrio species more frequently occur in warmer waters or in the seasons that the temperature of coastal waters is suitable for their growth. The risk of infection will be more when seafoods are consumed raw (Di Pinto *et al*., 2008), or in an insufficiently cooked mode and also when they are post-heat contaminated (Noorlis *et al*., 2011).

Vibrio species have been frequently associated in many food poisoning outbreaks and they are considered as one of the most important pathogens associated with foodborne and waterborne diseases. Figure 1.2 shows the results of a report published by Morbidity and Mortality Weekly Report (MMWR) on incidence and trends of infection with foodborne pathogens, obtained from 10 sites in the United States, since 2006 till 2014 (Centers for Disease Control and Prevention, 2015). As it is clearly observable, the population of *Vibrio* species has experienced an upward trend through these years. Specially, in the recent years, from 2012 till 2014, a drastic growth in the prevalence of *Vibrio* species has been observed.

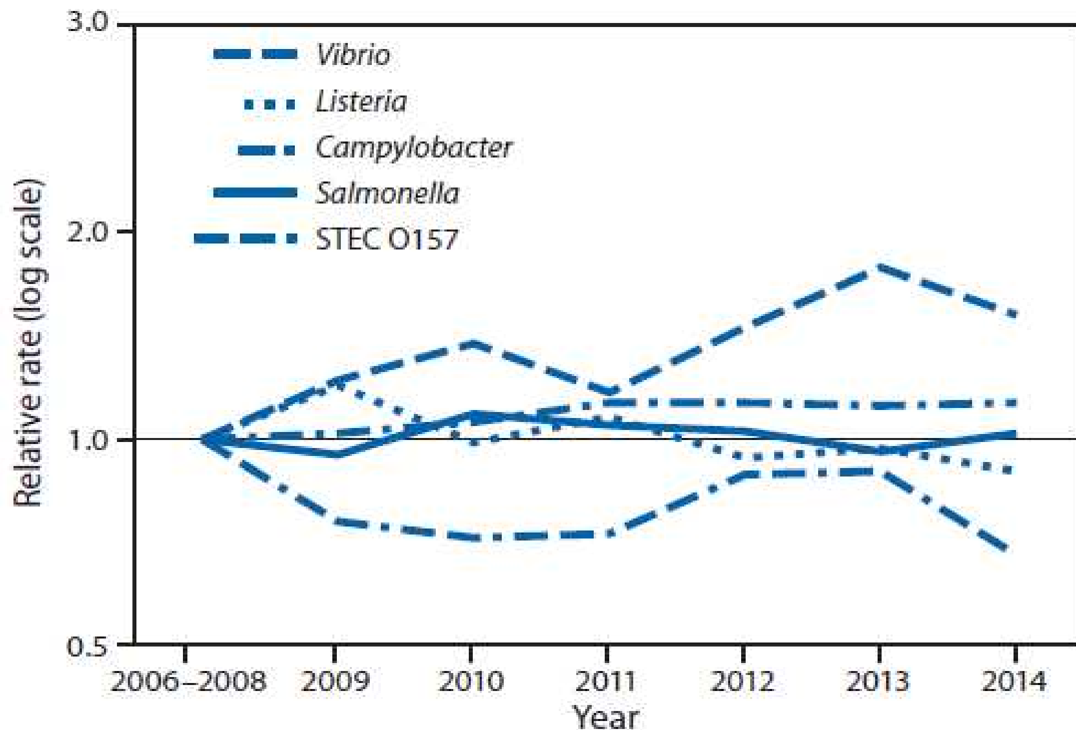


Figure 1.2: Relative rates of culture-confirmed infections with *Campylobacter*, STEC* O157, *Listeria*, *Salmonella*, and *Vibrio* compared with 2006–2008 rates, by year — Foodborne Diseases Active Surveillance Network, United States, 2006–2014 (Centers for Disease Control and Prevention, 2015). * Shiga toxin–producing *Escherichia coli*.

Vibrio species implicated in vibriosis that affect marine fishes are *V. alginolyticus, V. anguillarum, V. harveyi, V. ordalii, V. salmonicida, V. splendidus,* and *V. vulnificus. V. tapetis* and *V. furnissii* have also been isolated from moribund fish (Jensen *et al.,* 2003) and eels (Esteve 1995), respectively, but the true pathogenicity of these species for fish has still to be clearly established (Gomez-Gil *et al.,* 2014). Pathogenic vibrios are associated with acute bacterial septicemias or chronic focal lesions (Hjeltnes and Roberts 1993). *V. anguillarum* and *V. salmonicida* appear to be primary pathogens, whereas the other species may harbor certain virulent strains that affect organisms under stressing conditions, e.g., crowded and polluted environments. Chatterjee *et al.,* (2012) have described some disease causing Vibrio species for aquaculture that is presented in the following table 1.1.

Table 1.1: Diseases caused by *Vibrio* spp. in aquaculture

Vibrio spp.	Host organism	Disease
Vibrio harveyi	*Peneause monodon* (Tiger prawn) *Litopenaeus vannamei* (Whiteshrimp) *Epinephelus coioides* (Grouper) *Sulculus diversicolor* (Japanese abalone)	Luminescent vibriosis resulting in mass mortality Up to 85% mortality in nauplii Gastroenteritis followed by mass mortality Mass mortality
V. alginolyticus	*P. monodon* (Tiger prawn)	Shell disease
V. parahaemolyticus	*P. monodon* (Tiger prawn)	Red disease, up to 80% mortality
V. anguillarum	*Salmo salar L.*(Salmon), *Oncorhynchus mykiss* (Rainbow trout)	Vibriosis
V. vulnificus	*Oreochromis niloticus* (Nile tilapia), Eels	Vibriosis
V. ordalii	Salmonids	Vibriosis
V. salmonicida	Atlantic salmon, cod	Vibriosis
Moritella viscose (V. viscosus)	Atlantic salmon, cod	Vibriosis

Vibriosis caused by infection by *Vibrio* spp., is one of the most prevalent diseases in fishes and other aquaculture-reared organisms and is widely responsible for mortality in cultured aquaculture systems worldwide (Chen *et al.,* 2000; Lavilla-Pitogo *et al.,* 1998). The primary mode of infection in fish consists of penetration of bacterium to the host tissue mainly via chemotactic activity, followed by deployment of an iron-sequestering system, resulting in eventual damage to the fish by means of extracellular products i.e. haemolysin and proteases. In shrimp, the possible routes of infection are feed, gill, hepatopancreas etc. *Vibrios* colonized the host tissue of shrimps after crossing the epithelial cells (Martin-Laurent *et al.,* 2001). Table 1 lists some of the important aquaculture diseases caused by different *Vibrio* spp.

Modern intensive shrimp systems provide almost ideal conditions for the propagation of diseases like vibriosis (Zhang *et al.,* 2014). During outbreaks in larval and postlarval shrimp rearing, luminescent *V. harveyi, V. campbellii*, and probably *V. splendidus* have been isolated. During the grow-out part of the culture, many species have been reported as responsible for vibriosis, but only a few have actually been proven to be pathogens; all others are only members of the normal microbiota of the shrimp and the environment. Species where some strains have been proven to be pathogenic for shrimps are *V. parahaemolyticus, V. penaeicida*, and probably *V. harveyi*. Vibrios are considered opportunistic pathogens, but evidence suggests that some strains can be regarded as primary pathogens, especially in the case of *V. penaeicida*. Artemia spp. can also be susceptible to infection by Vibrios, as is the case of *V. proteolyticus* and strains of *V. parahaemolyticus* and *V. campbellii* (Gomez-Gil *et al.,* 2014).

Some vibrios may cause disease in both aquatic animals and humans. The human outbreaks, although low in number, typically involve wound infections and gastro-intestinal disease often with watery diarrhoea. In a minority of cases, for example *V. vulnificus*, there is good evidence to actually associate human infections with diseased animals. In other cases, the link is certainly feasible but hard evidence is mostly lacking. B. Austin (2010) categorizes *V. cholera, V. parahaemolyticus* and *V. vulnificus* as higher risk organisms for zoonoses, and *Grimontia (=Vibrio) hollisae, Photobacterium (=Vibrio) damselae* subsp. *damselae*, *V. alginolyticus, V. harveyi, V. fluvialis, V. furnissii, V. metschnikovii* and *V. mimicus* as lower risk organisms.

The following table 1.2 shows the summary of the above findings representing some Vibrio species and their relation to human infection.

Table 1.2 Summary of *Vibrio* spp. and human infection

Species	Human infection		
	Gastroenteritis /diarrhea	Wound/Ear	Septicemia
V. cholerae O1/O139	Yes	Yes	No
Non O1/O139	Yes	Yes	Rare
V. parahaemolyticus	Yes	Yes	Rare
V. vulnificus	Yes	Yes	Yes
V. mimicus	Yes	Rare	Rare
V. hollisae	Yes	Rare	Rare
V. fluvialis	Yes	Rare	Rare
V. alginolyticus	Yes	Yes	Yes
Photobacterium damsela	No	Yes	Yes
V. metschnikovii	Rare	Rare	Rare
V. cincinnatiensis	Rare	No	Rare
V. harveyi	No	Rare	No
V. furnissii	Rare	No	No

Highly occurrence of *Vibrio* spp. in marine and aquatic environments, leads to their presence in seafood and any food of freshwater origin, especially from temperate climates around the world. The abundance of *Vibrio* spp. in raw seafood and marine products makes these sorts of food appropriate for their transmission. And it results in the association of *Vibrio* spp. studies with food safety issues.

Recent ecological studies have shown that seasonal changes in coastal water bodies, e.g., temperature, lead to the predominance of different populations of *vibrio*s. *V. parahaemolyticus, V. campbellii and V. coralliilyticus* related species increase during the summer months, whereas *V. splendidus*- and *V. pectenicida*-like occur year-round (Thompson *et al.,* 2004).

Vibrios are fairly easy to isolate from both clinical and environmental materials, though some species may require growth factors and/or vitamins (Gomez-Gil *et al.,* 2014). Of special mention is the need of NaCl for most of the species of Vibrio, although some

species can grow with minimum NaCl concentrations, e.g., *V. cholerae, V. mimicus, V. hispanicus,* and some strains of *V. fluvialis, V. furnissii,* and *V. metschnikovii* (Alsina and Blanch 1994b; Gomez-Gil *et al.,* 2004). Optimal Na^+ concentration for many marine bacteria is between 70 and 300 mM (Reichelt and Baumann 1974). Different strategies may be used in order to isolate specific Vibrio species from environmental and clinical samples. The most common media used for the isolation and cultivation of vibrios are Thiosulfate–citrate–bile salts agar (TCBS), Trypticase Soy Medium (TSA and TSB for the agar and broth, respectively), Marine Agar, Alkaline Peptone Water (APW) etc.

Thiosulfate-citrate-bile salts-sucrose (TCBS) has been recommended in the standard method for the isolation of *V. cholerae* from foods. The method includes an enrichment in Alkaline Peptone Water (APW) at 35±2ºC overnight and then isolation on TCBS medium. The same method was also recommended for other Vibrios, such as *V. vulnificus* and *V. parahaemolyticus* as well (Elliot *et al*., 1995). Most *Vibrio* species have a considerable growth on TCBS while the growth of most non-vibrios is inhibited on this medium. However, *V. parahaemolyticus* colonies on TCBS are very difficult to distinguish visually from the colonies of other bacteria, because they might be covered by a yellow color produced by sucrose-fermenting bacteria (Hara-Kudo *et al*., 2001). The colony colours that appear on TCBS for different *Vibrio* species have been presented in table 1.3 and figure 1.3.

Table 1.3: Colony colors of *Vibrio* spp. on TCBS agar

Species	Colony Color
V. parahaemolyticus	Green
V. vulnificus	Green
V. cholerae	Yellow
V. alginolyticus	Yellow
V. furnissii	Yellow
V. fluvialis	Yellow

Source: Hardy Diagnostics (www.catalog.hardydiagnostics.com)

Vibrios represent a considerable fraction of the microbiota in rearing systems. The high density of animals and feeding loads applied favor a proliferation of vibrios in these settings. In addition, the use of antibiotics in the aquaculture industry is a well-known

but yet regrettable practice that probably increases the abundance of resistant strains of vibrios. In many developing countries, where the majority of aquaculture practices take place, there is no regulation, or where regulations exist, they are not enforced (Alderman and Hastings, 1998). This type of antibiotic abuse has led to the development of resistance, especially in microenvironments (Walsh, 2003).

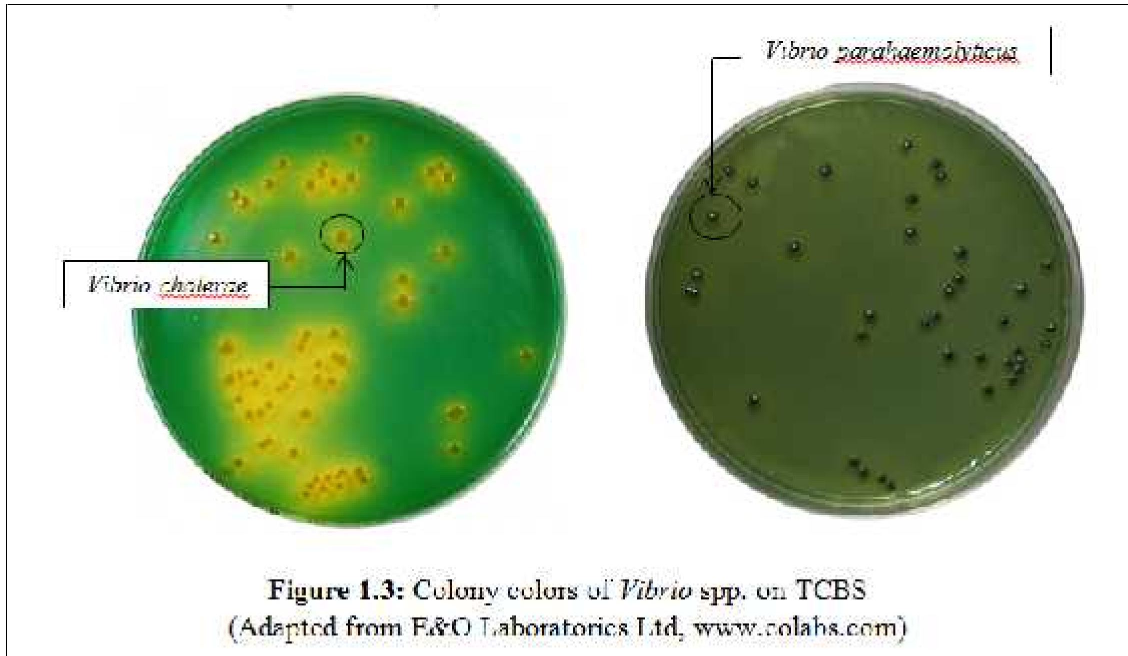


Figure 1.3: Colony colors of *Vibrio* spp. on TCBS
(Adapted from E&O Laboratories Ltd, www.eolabs.com)

Antibiotic resistance has been reported from many vibrios, but especially from isolates obtained from the aquaculture industry. Resistance to various antibiotics has been found in vibrios isolated from the marine environment (Pradeep and Lakshmanaperumalsamy 1985; Molitoris *et al.,* 1985), Artemia nauplii (Hameed and Balasubramanian 2000), penaeid shrimps (Bhattacharya *et al.,* 2000; Roque *et al.,* 2001; Molina-Aja *et al.,* 2002), fish (Austin *et al.,* 1982; Sanjeev and Stephen 1992; Miranda and Rojas 1996; Li-Jun *et al.,* 1999), and molluscs (Tubiash *et al.,* 1965; Martinez-Manzanares *et al.,* 1998).

The spread of antibiotic resistance among Vibrios has been documented to occur by transfer of plasmids that carry antibiotic resistance determinants (e.g., TEM or tet genes) between species or genera by conjugation (Aoki *et al.,* 1984; Li-Jun *et al.,* 1999; Molina-Aja *et al.,* 2002). In this study we observed the resistance in Vibrios to some of the most commonly used antibiotics in aquaculture.

Many groups of bacteria have been used as probiotics in human and farm animals. Potential probiotic bacteria have been used to combat Vibrios that affect marine cultured organisms and also Vibrios as probionts against other pathogenic Vibrios (Gomez-Gil *et al.,* 2000; Hjelm Mette *et al.,* 2004). *V. alginolyticus* is probably the species most studied as a potential probiont. This organism has been found as the dominant species in healthy cultures of rotifers and turbot (*Scophthalmus maximus*) larvae (Gatesoupe 1990) and as a growth promoter of rotifers (*Brachionus plicatilis*) (Bogaert *et al.,* 1993). *V. alginolyticus* has been found to be more abundant in the intestine of healthy fish larvae than in those where mortality outbreaks have been observed (Tanasomwang and Muroga 1988; Verdonck *et al.,* 1997; Grisez *et al.,* 1997), suggesting that this species protects fish larvae against the colonization of potential pathogens. The application of a strain identified as *V. alginolyticus* to Atlantic salmon (*Salmo salar*) 7 days prior to the addition of pathogenic bacteria reduced the mortalities to up to 100 % when the fish were challenged with *Aeromonas salmonicida*, *V. anguillarum*, and *V. ordalii* (Austin *et al.,* 1995). A strain of *V. alginolyticus*-like, introduced via the rotifer (*Brachionus plicatilis*) into turbot (*Scophthalmus maximus*) larvae, reduced the mortality of experimentally infected fish to up to 80 %, (Gatesoupe 1997). This species also works again *V. parahaemolyticus* (Gomez-Gil 1998), *V. proteolyticus* (Verschuere *et al.,* 2000). Besides these, *V. Pelagius* (Ringo and Vadstein 1998) and some other vibrios like *Vibrio mediterranei* (Huys *et al.,* 2001) are potential probionts.

A key step in understanding microbial community structure, dynamics, and how organisms might influence or be influenced by their surroundings is to classify DNA sequences taxonomically or phylogenetically (Poretsky *et al.,* 2014). To date, most studies of microbial communities in systems ranging from the open ocean to soil to the human gut have depended on a single gene, the 16S small subunit ribosomal RNA (rRNA) gene (Costello *et al.,* 2009; Gilbert *et al.,* 2012; Kent *et al.,* 2004; Nemergut *et al.,* 2011). Massively parallel sequencing methods are increasingly being applied to the characterization of microbial communities based on amplification of this gene and have led to a better appreciation of extant biodiversity (Sogin *et al.,* 2006).

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. For many years, sequencing of the 16S ribosomal RNA (rRNA) gene has served as an important tool for

determining phylogenetic relationships between bacteria. The 16S rRNA gene is a section of prokaryotic DNA found in all bacteria and archaea. The features of this molecular target that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, and can lead to the recognition of novel pathogens and noncultured bacteria. In clinical microbiology, molecular identification based on 16S rRNA sequencing is applied fundamentally to bacteria whose identification by means of other types of techniques is impossible or difficult (Matsumoto *et al.,* 2013).

For bacterial identification, 16S rRNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections. Not only has it provided insights into aetiologies of infectious disease, but it also helps clinicians in choosing antibiotics and in determining the duration of treatment and infection control procedures (Woo *et al.,* 2008).

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (Janda *et al.,* 2007) (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

A molecular technique based on the restriction fragment length polymorphism (RFLP) of the 16S ribosomal genes amplified by a polymerase chain reaction (PCR), referred to as amplified 16S ribosomal DNA restriction analysis (ARDRA), has been developed as a practical short cut to full sequence determination. By using ARDRA, now, when new species are described, there is no need to develop new probes or primers. Instead, new ARDRA profiles can be easily added to the existing library. Also, ARDRA profiles for newly described species can be predicted by applying computer aided digestion of the available GenBank sequences, given the availability of sequences of sufficient quality (Turenne *et al.,* 2001).

ARDRA is commonly utilized as an alternative to more laborious and expensive methods for the identification of eubacteria, being the analysis of the rRNA cistron a good criterion for microbial classification at both genus and species level (Grimont and Grimont, 1986; Massol-Deya *et al.,*, 1995). For amplified ribosomal DNA restriction analysis, PCR-amplified 16S rRNA fragments are digested or cut at specific sites with restriction enzymes and the resulting digest separated by gel electrophoresis.

However, ARDRA is useful for detecting structural changes in microbial communities but is unable to measure microbial diversity or detection of specific phylogenetic groups within a community fingerprinting profile (Liu *et al.,*, 1997). Optimization with restriction enzymes is required and is often difficult if sequences are unknown. As a result, further optimization may be required to produce fingerprinting patterns characteristics of the microbial community (Vaneechoutte *et al.,*, 1992; Spiegelman *et al.,*, 2005). In addition, banding patterns in diverse communities become too complex to analyze using ARDRA (Kirk *et al.,*, 2004).

Species-specific identification using phenotypic characterization is still available for phenotypically distinct species with major modification on the use of commercially available kit (API 20E, Crystal E/NF, BIOLOG GN2, etc.) (O'Hara *et al.,* 2003). PCR-based methodologies and extended database of gene sequences provide rapid and reliable methods to identify bacterial species based on rather stable genetic elements than phenotypic traits. Nowadays, various kinds of group-selective, group-specific, and/or species-specific identification systems have been developed and used to study ecology of the members of *Vibrionaceae* (Nishibuchi 2006).

The phylogenetic structure of Vibrios has been laid in the early 1990s (Dorsch *et al.,* 1992; Kita-Tsukamoto *et al.,* 1993; Ruimy *et al.,* 1994). The almost complete 16S rRNA sequences of 10 Vibrio species revealed a *Vibrio* core group (*V. harveyi*-related species) and also showed that *V. hollisae* should be allocated into a new genus (Dorsch *et al.,* 1992). A comprehensive phylogenetic study of the *Vibrionaceae* was accomplished by Kita-Tsukamoto (Kita-Tsukamoto *et al.,* 1993) and coworkers. They sequenced a fragment of the 16S rRNA sequences (around 450 nt) of 50 species, including most known Vibrios, and species of *Aeromonas, Deleya, Escherichia, Marinomonas, Pseudomonas,* and *Shewanella.* The main outcomes of this study were (i) the

circumscription of species (at least 99.3 %16S rRNA similarity), genera (95–96 %), and family (90–91 %) borders within the *Vibrionaceae* and (ii) the delineation of seven main groups of *Vibrionaceae* species that would correspond to different genera or families. Subsequently, *V. costicola* was transferred into *Salinivibrio costicola* (Mellado *et al.,* 1996), *V. marinus* into *Moritella marina* (Urakawa *et al.,* 1999), and *V. iliopiscarius* into *Photobacterium iliopiscarius* (Urakawa *et al.,* 1998).*V. hollisae* was transferred into *Grimontia hollisae* (Thompson *et al.,* 2003). Genus "*Listonella"* is proposed as a later heterotrophic synonym based on the 16S rRNA gene phylogeny and genome features (Thompson *et al.,* 2011), and now *V. anguillarum* and *V. plagius* should be used in place of *L. anguillarum* and *L. pelagia*.

A consensus view emerged from these studies: Vibrios were highly heterogeneous. According to Kita-Tsukamoto, *V. cholera* and *V. mimicus* would correspond to a genus on their own (Kita-Tsukamoto *et al.,* 1993). *V. fischeri, V. logei, V. salmonicida,* and relatives should be elevated to the genus rank. In both cases, the status of these Vibrio species has not yet been fully determined. If *V. cholerae* and *V. mimicus* and the *V. fischeri*-related group are to be elevated to the genus level, then one might argue the revival of Beneckea to encompass all other remaining Vibrios, an idea which was originally laid down by Bauman (Allen and Baumann 1971). As recently *V. fischeri, V. logei, V. salmonicida,* and *V. wodanis* are transferred into *Aliivibrio* gen. nov. by 16S rRNA gene phylogeny, MLSA, and phenotypic characterization (Urbanczyk *et al.,* 2007); more accurate phylogenetic pictures for *Vibrionaceae* must be reconstructed.

The most recent phylogenetic tree for all currently known 131 *Vibrionaceae* species and one describing species (*Vibrio tritonius* sp. nov.) based on 16S rRNA gene according to the ALL-Species Living Tree Project (LTP) database (Yarza *et al.,* 2010) is described by Gomez-Gil *et al.,* (2014). All species are belonged into a single cluster forming *Vibrionaceae* on the basis of the well-cured LTP database and neighbor-joining algorithm. The family *Vibrionaceae* is moderately related to the family *Enterobacteriaceae*. *Shewanellaceae, Pseudoalteromonadaceae, Aeromonadaceae, Pasteurellaceae,* and *Succinivibrionaceae* appear as sister clades of these two families, while clades of *Alteromonadacea* and *Idiomarinaceae* are branching slightly deeper. In more detail pictures of these genera, each genus of *Photobacterium, Enterovibrio, Grimontia, and Salinivibrio* seems to form each cluster. However, the genus *Aliivibrio* is

nested within the cluster of the genus *Vibrio*; nevertheless, *V. fischeri, V. logei, V. salmonicida,* and *V. wodanis* are reclassified as *Aliivibrio* gen. nov. recently (Urbanczyk *et al.,* 2007). In further analysis on the phylogeny of the genus *Aliivibrio* using the latest 16S rRNA gene sequence data set, the genus formed a robust clade (>99 % bootstrap support by NJ; MP and ML), but it is located at the terminal branch of *Vibrio halioticoli* and/or the related species clusters. So, on the basis of the 16S rRNA gene tool, it is hard to say each genus in *Vibrionaceae* is supported as a robust clade; it seems to be polyphyletic.

Sawabe *et al.* (2007) propose the use of a split decomposition analysis using multilocus gene sequence data set to define more robust clades in *Vibrionaceae*. Based on nine genes (i.e., *fts*A, *gap*A, *gyr*B, *mre*B, *pyr*H, *rec*A, *rpo*A, *top*A, and 16S rRNA) multilocus sequence analysis (MLSA) of 78 type strains belonged to *Vibrionaceae*; at least 14 monophyletic clades are found with a significant bootstrap support. The species within each clade shared >20% DDH, <5 % GC variation (mol %), >85 % MLSA sequence similarity, and >89 % average amino acid identity (AAI) (Sawabe *et al.,* 2007). The analysis is capable of an elucidating minimum evolutionary unit in *Vibrionaceae* as the "clade," but more robust phylogenetic relationships among the clades are remained to be veiled. The introduction of MLSA or genome base taxonomy on Vibrionaceae could make new promising future insights into the better and fine-scale solutions in the vibrio taxonomy and phylogeny.

1.2 Rationale

As fisheries production and intensification of Bangladesh have boomed, so too have the disease prevalence. This giant sector has been stumbled adversely. A crisis in the shrimp industry over the last few years is due to largely to an increase in virulence of pathogens, especially bacterial diseases caused by Vibrio spp, together with white spot viruses (Rahman *et al.,* 2010). According to a World Bank report that estimated the global losses due to shrimp diseases are around US$ 3 billion(Lundin *et al.,* 2006) Fisheries in both salt water and freshwater are becoming increasingly vulnerable to bacterial infection due to the ease with which pathogens are transmitted in aquaculture(Boaventura *et al.,* 2006). Nevertheless disease outbreaks are being increasingly recognized as a noteworthy impediment on aquaculture production and trade, affecting the economic development of the sector in Bangladesh like many other countries. Various infectious diseases caused

by bacteria, virus and protozoa are now a primary concern in aquaculture (Rahman *et al.,* 2014). Diseases caused by *Vibrio* spp. and *Aeromonas* spp. are commonly implicated in episodes of mortality (Watson *et al.,* 2008). For instance vibriosis is currently one of the main diseases affecting shrimp culture and outbreaks lead to dramatic crop failures in the major shrimp producing countries (Lightner 1988; Lin 1995).

Among possible sources of bacterial infections, *Vibrio* species are one of the deadliest fish and shrimp pathogen that cause heavy mortalities in aquaculture industries worldwide (Lightner & Lewis 1975, Nishibuchi *et al.,* 1991, Bondad-Reantaso *et al.,* 2005). Most of the *vibrio* species are pathogenic to humans and are usually responsible for causing alimentary infections in countries with warm coastal waters, where fish and shrimp are consumed raw or lightly cooked (Jaksic *et al.* 2002; Messelhäusser *et al.* 2010). Among them *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are the most important ones, with a worldwide distribution (Gomez-Gil *et al.,* 2014).

The common pathogenic *vibrio* species for aquaculture are *Vibrio alginolyticus, Vibrio anguillarum, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio campbellii, Vibrio splendidus, Vibrio penaeicida, Vibrio nigripulchritudo* and *Vibrio vulnificus* etc. These pathogens cause serious infections, decreased production both in the hatchery and grow-out ponds, reduced feed conversion and growth rates in surviving individuals, thus having a negative impact on the overall financial efficiency of the business. Vibriosis has been the main cause of production loss due to bacterial disease in shrimp farms in south Iran in recent years (Hosseini *et al.,* 2004), and many other South Asian countries too. High prevalence of *Vibrio* spp. in coastal waters and seafood products of Southeast Asian countries has been reported by many investigators (Wong *et al.,* 1999; Zulkifli *et al.*, 2009), due to the optimal condition for their growth. Moreover, the consumption of seafoods and marine products is quite high in these countries, and consequently there is a high risk of infection and diseases associated with *Vibrio* species in Malaysia and other Southeast Asian countries. Consequently, efficient methods for detection, differentiation and characterization of *Vibrio* spp. are required to be included in screening programs in order to prevent infections and diseases associated with the pathogenic strains. However, there is no extensive study on the prevalence of *Vibrio* spp. in Bangladesh, except *V. cholerae.*

Hatchery owners of Bangladesh often suffer hectic economic losses due to mass mortality of fish and shrimps by vibriosis, EMS (Early Mortality Syndrome) and many like other diseases, but in most of the time the causes remain unknown to them. The consumers are also unaware about the prevalence and pathogenicity of this group of bacteria. Therefore, it is of utmost importance to detect the prevalence of this community in shrimp and fish hatcheries as basis for preventive protection policy to hatcheries and farms as well as public health concern.

Identification of the causative organisms up to the species level is very useful for research and epidemiological studies as it helps in determining the exact source of any outbreak and in devising strategies to reduce the severity of the disease. However, the conventional identification techniques involving a series of biochemical tests and agglutination with specific antisera are time consuming and ambiguous (Sakazaki, 1992). Therefore, in this study we have characterized the sample isolates by 16S rRNA gene sequencing for species-specific identification of Vibrios.

1.3 Objectives

The overall goal of this study is to understand the prevalence and vibrosis caused by *Vibrio* community in the sampled fish and shrimp hatcheries of Bangladesh.

The specific objectives are:

- to measure the microbial load in the samples;
- to isolate *Vibrio* species from the sample;
- to identify different species/strains of this community in the studied area;
- to amplify with the universal primers, the 16S rRNA from the Vibrio strains
- to construct phylogenetic tree for better understanding of the relations among the species.

Chapter 2

Materials and Methods

2.1 Sampling

A total of 43 samples, 16 of which are from coastal shrimp hatcheries and the rest are from freshwater fish hatcheries, have been collected and examined. The sampling was done during the period of June 2015 to August 2015. The samples include shrimp Postlarvae (PL), artemia (live feed) nauplii, fry of different cultured species of fish and the cultured water. Samples were collected from three different districts of Bangladesh- Cox's Bazar, Mymensingh and Bogra. Even though the distribution of *Vibrio* spp is mainly in salt water and occasionally in freshwater, we studied the both with equal emphasis as Bangladesh is currently the fifth largest freshwater fish producing country in the world (FAO Agricultural Statistical Handbook, 2015).

Table 2.1: List of samples collected from Cox's Bazar

Sample Number	Sample	Hatchery
C1	Artemia nauplii from Tank 1	Zomzom Hatchery Ltd
C2	Artemia nauplii from Tank 2	Zomzom Hatchery Ltd
C3	Shrimp PL of 10 days, Tank 1	Zomzom Hatchery Ltd
C4	Water from PL(10) Tank 1	Zomzom Hatchery Ltd
C5	Shrimp PL of 10 days, Tank 2	Zomzom Hatchery Ltd
C6	Water from PL(10) Tank 2	Zomzom Hatchery Ltd
C7	Shrimp PL of 12 days	Zomzom Hatchery Ltd
C8	Water from PL(12) Tank	Zomzom Hatchery Ltd
C9	Artemia nauplii from Tank 1	Zomzom Hatchery Ltd
C10	Artemia nauplii from Tank 2	Zomzom Hatchery Ltd
C11	Shrimp PL of 8 days	Zomzom Hatchery Ltd
C12	Water from PL(8) Tank	Zomzom Hatchery Ltd
C13	Shrimp PL of 12 days	Zomzom Hatchery Ltd
C14	Water from PL(12) Tank	Zomzom Hatchery Ltd
C15	Shrimp PL of 10 days	Zomzom Hatchery Ltd
C16	Water from PL(10) Tank	Zomzom Hatchery Ltd

Table 2.2: List of samples collected from Trishal, Mymensingh

Sample No.	Sample	Hatchery
M1	Tilapia fry, 40 days, Big size	Reliance Hatchery Ltd
M2	Tilapia fry, 24 days, Medium	Reliance Hatchery Ltd
M3	Tilapia fry, 40 days, Small size	Reliance Hatchery Ltd
M4	Tilapia fry, 25 days	Reliance Hatchery Ltd
M5	Tilapia fry, 25 days	Reliance Hatchery Ltd
M6	Tilapia fry, 25days	Reliance Hatchery Ltd
M7	Tilapia fry, 28days	Reliance Hatchery Ltd
M8	Tilapia fry, 28days	Reliance Hatchery Ltd
M9	Tilapia fry, 28days	Reliance Hatchery Ltd
M10	Tilapia fry, 33days	Reliance Hatchery Ltd
M11	Tilapia fry, 33days	Reliance Hatchery Ltd
M12	Tilapia fry, 33days	Reliance Hatchery Ltd
M13	Water from the hapa- Tilapia of 25 days	Reliance Hatchery Ltd
M14	Water from the hapa- Tilapia of 25 days	Reliance Hatchery Ltd

Table 2.3: List of samples collected from Adamdighi, Boagra

Sample No.	Sample	Hatchery
B1	Magur fry, 5 days	G.M. Aquaculture Ltd
B2	Water from the tank of Magur 5 days	G.M. Aquaculture Ltd
B3	Water from live feed pond	G.M. Aquaculture Ltd

B4	Golsha fry, 2 days	G.M. Aquaculture Ltd
B5	Water from the tank of Magur 5 days	G.M. Aquaculture Ltd
B6	Shing fry, 6 days	G.M. Aquaculture Ltd
B7	Water from the tank of Magur 5 days	G.M. Aquaculture Ltd
B8	Magur fry, 6 days	G.M. Aquaculture Ltd
B9	Water from the tank of Magur 5 days	G.M. Aquaculture Ltd
B10	Pangas fry, 5 days	G.M. Aquaculture Ltd
B11	Water from the tank of Magur 5 days	G.M. Aquaculture Ltd
B12	Pangas fry, 1 day	G.M. Aquaculture Ltd
B13	Water from the tank of Pangas 1 day	G.M. Aquaculture Ltd
B14	Water from live feed pond	G.M. Aquaculture Ltd

2.2 Description of the sampling areas

2.2.1 Cox’s Bazar

Cox’s Bazar is one of the largest fish and shrimp producing districts in Bangladesh and is bestowed by the touch of the Bay of Bengal. The annual average temperature in Cox's Bazar remains at about a maximum of 34.8 °C and a minimum of 16.1 °C. The average amount of rainfall remains at 4,285 mm. A number of shrimp hatcheries are established in this region, the Zamzam Hatchery Ltd and the Balaka Hatchery Ltd are notable two.

2.2.2 Mymensingh

Mymensingh is a region where freshwater fish culture is widely practiced. This district supplies a huge proportion of fish in the country market. Many fish culture farm and hatcheries are situated in this region. The Reliance Hatchery Ltd and the Agro 3 Hatchery Ltd of Trishal Upazila are two hatcheries which practice standard procedures of fish culture.

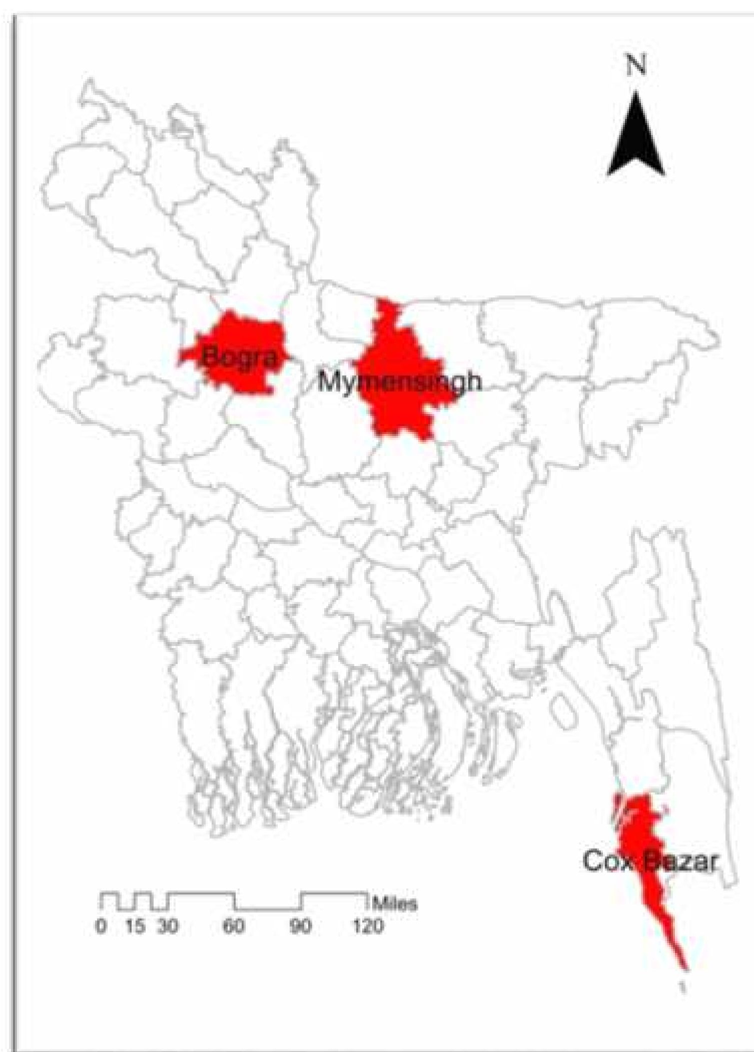


Figure 2.1: Location of sampling areas

2.2.3 Bogra

The Adamdighi upazila of the district Bogra is well-known for its fish and fry production in the country. There are many small, medium and large scale fish farm and hatcheries in this region. The representatives are G.M. Aquaculture Ltd, Mandal Fish Farm & Hatchery, Khan Fish Farm & Hatchery and Rafi Fish Farm & Hatchery Ltd.

2.3 Laboratories of investigation

All the studies were carried out in the Aquatic Laboratory of Department of Fisheries in collaboration with Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka. The sequencing was done in the First BASE Laboratories Sdn Bhd, Malaysia.

2.4 Sample collection

The samples were collected from the nursery tank using scoop net and bowl. Then were kept in icebox maintaining temperature at -4^{0}C and kept at -20^{0}C after transferred in the laboratory.

All samples were collected following the method of American Public Health Association (APHA, 1970, 1998).

2.5 Sample preparation

Fry and PL samples were aseptically grinded in a mortar and blended with physiological saline (0.85% NaCl). The water samples were kept just as it was. All blended samples were kept in a distance to reduce cross contamination.

2.6 Bacterial enumeration

2.6.1 Total bacterial colony Count (TBC)

Serial dilution technique (Greenberg *et al.,* 1980) was used for counting the bacterial colonies easily. 100 µL blended solution was mixed with 900 µL of sterile saline water in an eppendorf using vortex machine. This process was repeated three more times to get the final 4th dilution from which 100 µL solution was spread in Nutrient Agar (NA) plate and then the plates were kept at 37^{0}C for 24 hours in the incubator. After this time, the number of bacterial colonies grown in the NA media was counted.

The same procedure was followed for total bacterial count in Marine Agar (MA) Plates.

2.6.2 Total *Vibrio* like colonies count

100 µL blended raw solution from each sample was spread in Thiosulfate Citrate Bile Salt Sucrose agar (TCBS) plate and then the plates were kept at 37^{0}C for incubation for 24 hours. Then the *vibrio* colony count was taken from these TCBS plates.

2.7 Isolation of bacteria

2.7.1 Enrichment in Alkaline Peptone Water (APW)

Alkaline Peptone Water was used for the enrichment of the samples in order to provide a suitable environment for *Vibrio* spp. to grow and reach to a detectable level for the presumptive identification. 1 mL blended solution from each sample was taken in 9 mL

alkaline peptone water in a test tube. These tubes were kept in incubator at 37^0C for 6 to 24 hours.

2.7.2 Bacterial isolation

After the incubation period some solution (bacteria) were transferred from APW tube to TCBS agar media and Nutrient Agar media aseptically using loop and then streaked. The streaked TCBS and NA plates were kept in incubator at 37^0C for 24 hours.

2.7.3 Single colony subculture

Bacterial colonies grew in the TCBS and NA plates and after observing their morphology only single colonies with dissimilar traits were picked and then subcultured in new TCBS and NA plates.

2.8 Preservation of isolates

The subcultured single colonies were further screened on their morphological appearance and only variant isolates were stored in Luria-Bertani (LB) broth supplemented with 10% glycerol at -80^0C for future use.

2.9 Molecular analysis of the isolates for identification

Chromosomal DNA of the selected 37 isolates was extracted followed by amplification of the 16S rRNA gene of the isolates by Polymerase Chain Reaction (PCR) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) to group the isolates into different genotypes. 16S rRNA gene amplicons of selected isolates representative of each genotype were sequenced followed by phylogenetic analysis to find out their close relatives.

2.9.1 Boiled template preparation for PCR (DNA Extraction)

Protocol of boiling method for bacterial chromosomal DNA extraction:

1. Pure bacteria culture in Nutrient Agar/ TCBS
2. Subculture in broth media
3. 1mL broth culture taken in eppendorf tube and centrifused at 10000 rpm for five minutes
4. Supernatant discarded and pellet collected
5. 200 µL of Tris-EDTA (TE) buffer was added in pellet and dissolved by finger shaking and vortex

6. Eppendorf cap was pierced by sterile needle
7. Boiling the eppendorf tube in dish with distilled water at 100ºC for ten minutes by gas burner
8. Just after boiling, eppendorf tubes were placed in ice for 10 minutes and cooled down
9. Centrifugation at 10000 rpm for ten minutes
10. Collection of 100-150 µL supernatant containing bacterial chromosomal DNA in fresh micro centrifuge tube (MCT) used as template for PCR.

2.9.2 Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA gene in the selected isolates was carried out by polymerase chain reaction (PCR) for further analysis.

2.9.2.1 Preparation of Reaction Mixture

The reaction mixture for PCR was prepared by mixing the specific volume of the components in an appropriate sized tube in the order provided in the following table 2.5. For a large number of reactions, a master mix without any template DNA was prepared and aliquoted into PCR tubes. At the end, specific template was added into a properly labeled PCR tube. The PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifugation briefly to mix gently and collect all components to the bottom of the tube respectively. In all PCR, a negative control that contained no DNA template but all other components of the reaction was included and in relevant cases, a positive control that contained known DNA template carrying known gene was also included. The PCR tubes were then placed in a thermal cycler (Veriti 96-Well Thermal Cycler, Applied Biosciences, USA) and the amplification parameters were set correctly.

Table 2.4: Universal primers of 16s rRNA used in PCR

Primer name	Type	Sequence (5'-3')	Reference
27F	Forward	AGAGTTTGATCMTGGCTCAG	Lane 1991
1492R	Reverse	TACCTTGTTACGACTT	Frank *et al.,* 2008

Table 2.5: Components of the reaction mixture for PCR

Component	Amount (for 1 Reaction)	Total amount (for 20 Reactions)
Master Mix GoTaq® (2X)	7.5 µL	7.5× 20= 150 µL
Nuclease Free Water	4.5 µL	4.5×20= 90 µL
Forward Primer	0.75 µL	0.75×20= 15 µL
Reverse Primer	0.75 µL	0.75×20= 15 µL
Template DNA	1.5 µL	1.5×20= 30 µL
Total Reaction Volume	15 µL	15×20= 300 µL

2.9.2.2 PCR Conditions

All the PCR tubes containing the reaction mixtures were heated at 94ºC for 5 minutes in the thermal cycler to ensure the denaturation of all DNA templates. The PCR reaction was then continued according to the following program:

Step 1: Denaturation at 94ºC for 1 minute
Step 2: Annealing at 57°C for 1 min

Step 3: Extension at 72ºC, for 45 second- 1 minute 45 second depending on the size of the target.

These three steps were repeated sequentially for 40 cycles with a final extension for 7 minutes at 72ºC. After completion of the reaction, PCR tubes were stored at -20ºC until further analysis. The cycling profile for each primer-target combination was optimized accordingly.

2.9.3 Analysis of the amplicons by agarose gel electrophoresis

The successful amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel (w/v) depending on the size of amplicon. Specific amount of agarose (Sigma, USA) was added in 1X Tris-acetate EDTA (TAE) buffer to prepare a desired final concentration of agarose in a final volume of 60 mL and was heated in a microwave oven for about 3 minutes to dissolve the agarose. The boiled mixture was allowed to cool to about 45°C and Ethidium Bromide (EtBr) was added to a final concentration of 0.5 µg/mL. The gel was poured onto gel casing preset with well former

(comb). The casing was then allowed to set on a flat surface for about 15 minutes. After solidification of the gel, the comb was removed and buffer (1x TAE) was poured into tank to submerge the solidified gel. Samples were prepared by mixing 5µl PCR product with 1µl 6X loading buffer. Molecular weight marker was prepared by mixing 5µl molecular weight marker with 1µl 6x loading buffer. Samples were loaded into the wells formed in the gel. Electrophoresis was operated at 100 volts for 35-70 minutes as per requirement. The gel was viewed using Alpha Imager HP Gel-documentation system (Cell Bioscience, USA).

2.9.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Complete digestion of 16S rRNA gene amplicons of the 38 presumptive vibrio isolates was done using the *Alu*I (Promega, USA) restriction enzyme. The restriction digestion (20 µL of final volume) was carried out for 4 hours at 37°C. The preparation for the reaction was done following the table 2.6. The resulting digestion products were resolved by agarose gel electrophoresis previously described using 2% agarose (w/v) gel running for 125 minutes at 70V.

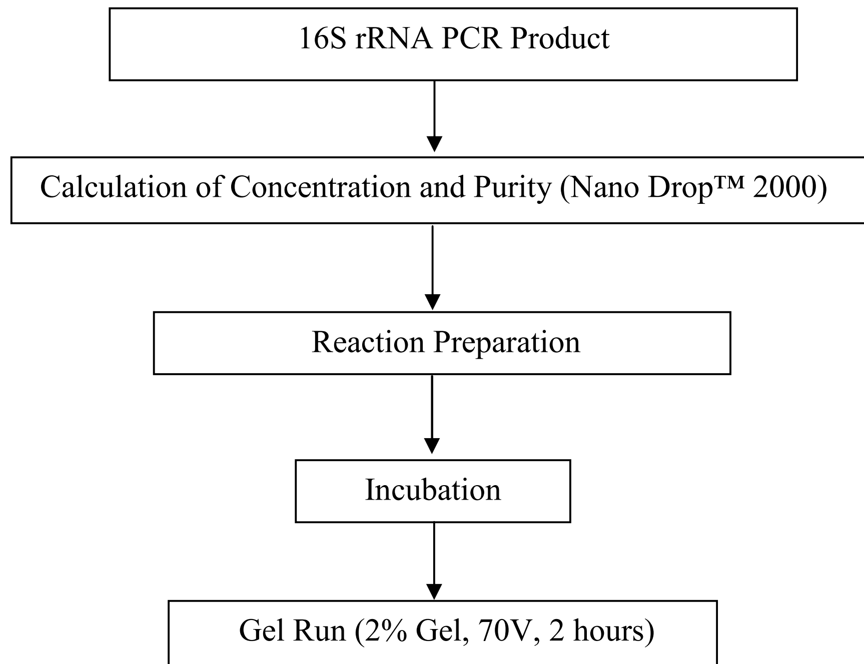
a. uncut experimental DNA,
b. digestion of commercially supplied control DNA and
c. no-enzyme "mock" digestion.

1 kb (Promega, USA) DNA ladders were used to analyze different restriction fragments.

Table 2.6: Reaction Preparation for ARDRA

Component	Amount (for 1 Reaction)	Final Concentration (for 38 Reactions)
***Alu*I restriction enzyme**	0.5 µL	0.5×38= 19 µL
Reaction Buffer(10x)	2 µL	2×38= 76 µL
Template	3 µL	3×38= 114 µL
Nuclease Free Water	14.5 µL	14.5×38=551 µL
Total	20 µL	20×38= 760 µL

Flowchart A: ARDRA protocol



2.9.5 Purification of PCR products and sequencing

The PCR products were resolved by agarose gel electrophoresis to confirm successful amplification of the desired sequence. If any nonspecific band existed, the specific band was excised from the gel and purified. The PCR products of specific genes were purified with the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instruction prior to sequencing. The steps of purification are given below in brief:

1) Processing of PCR amplifications

I. An equal volume of Membrane Binding Solution was added to the PCR amplification.

2) Binding of DNA:

I. A SV Minicolumn was inserted into Collection Tube

II. The dissolved gel mixture of prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute.

III. The preparation was centrifuged at 16,000 × *g* for 1 minute using the centrifuge (Sigma, USA). The flowthrough was discarded and the Minicolumn was reinserted into Collection Tube.

3) Washing:

I. 700µL Membrane Wash Solution (ethanol added) was added into the Minicolumn and centrifuged at 16,000 *g* for 1 minute. The flow-through was discarded and the Mini column was reinserted into Collection Tube.

II. The previous step was repeated with 500µL Membrane Wash Solution and centrifuged at 16,000 × *g* for 5 minutes.

III. The Collection Tube was emptied and the column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open (or off) to allow evaporation of any residual ethanol.

4) Elution:

I. The Minicolumn was carefully transferred to a clean 1.5mL micro centrifuge tube. 50µL Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1 minute followed by centrifugation at 16,000 × *g* for 1 minute.

II. The Minicolumn was discarded and DNA was stored at 4°C or –20°C.

2.9.6 16S rRNA sequencing

The PCR products were sent to First BASE Laboratories Sdn Bhd (Malaysia) where cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, USA) according to manufacturer's instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). Bidirectional (5′ to 3′ and 3′ to 5′) sequences were done for all the 8 representative isolates.

The basic sequencing protocol involved amplification of target sequence by PCR and purification of desired amplicon followed by cycle sequencing reaction, cycle sequencing product purification and capillary electrophoresis.

2.10 Chromatogram study

Chromatogram study (Figure 2.2) of the sequences was done using the software FinchTV Version 1.4 (Geospiza, Inc.). This study helped to trim the sequences from bidirectional sequencing output.

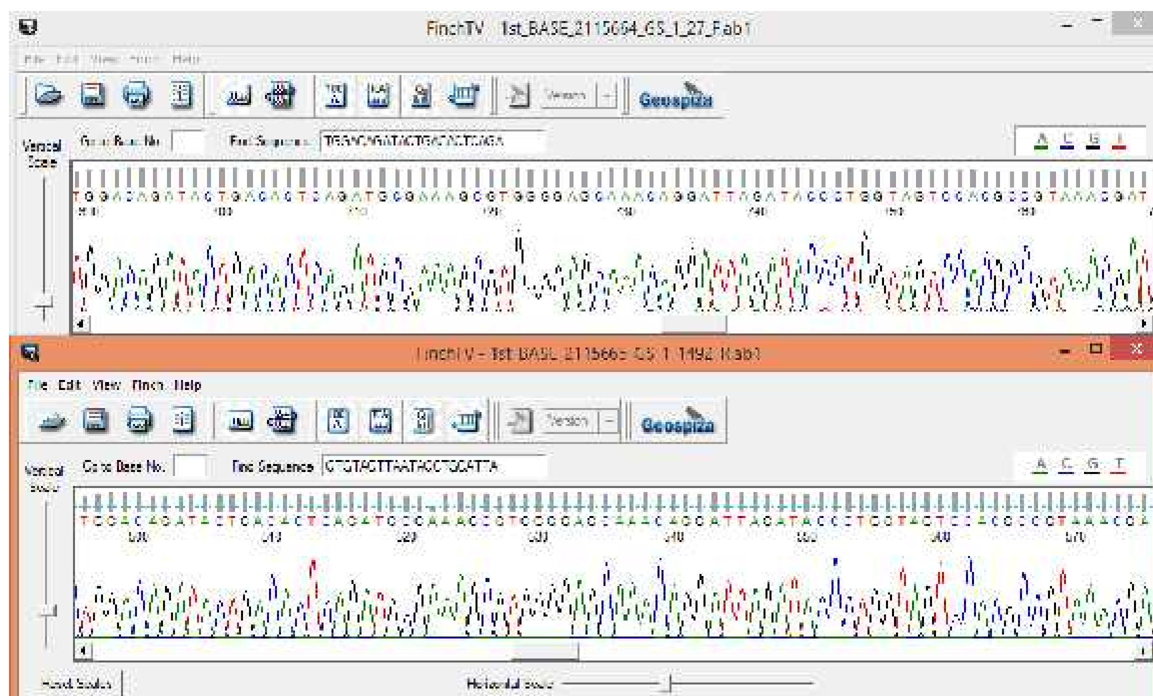


Figure 2.2: Chromatogram of the forward (F) and reverse (R) sequence of isolate arh1 in FinchTV

2.11 Identification of 16S rRNA gene sequence

The 16S rRNA sequences determined were compared with other sequences using Nucleotide BLAST ((http://blast.ncbi.nlm.nih.gov) program in NCBI (National Center for Biotechnology Information) determine the closest matching sequences in GenBank. From the scoring of relatedness shown in the search result, the best matched strains were considered as the identified isolates.

2.12 Multiple sequence alignment

Multiple sequence alignment was done for the sequenced isolate using the online program CLUSTALW (Thompson *et al.*1994) through NPS (Network for Protein Synthesis). For the nucleotide alignment, 1320 base pair sequences of each isolates were taken.

2.13 Phylogenetic analysis

These 16S rRNA sequences (the determined and reference sequences) were aligned using Interspecies/interstrain similarity for each gene was determined using Molecular Evolutionary Genetics Analysis (MEGA) Version 6.0 software (Tamura *et al.* 2013). Distance matrices were calculated using Kimura's 2-parameter distances. Robustness of

topologies was assessed by the bootstrap method with 1,000 replicates. Final classification of 16S rRNA gene to a phylogenetic division or subdivision was based on combined results from the phylogenetic group represented by the closest matching sequences in the GenBank and phylogenetic tree analyses.

Table 2.7: Four reference strains downloaded from NCBI GenBank to support the phylogenetic analysis

Reference Isolates	Gene Bank Accession No.
Vibrio rotiferianus partial 16S rRNA gene, strain LPD 1-1-86	FM204864.1
Vibrio campbellii partial 16S rRNA gene, strain R1311	FM204856.1
Vibrio parahaemolyticus partial 16S rRNA gene, CECT 611	FM204867.1
Vibrio ordalii gene for 16S rRNA, partial sequence	AB497069.1

2.14 Statistical analysis

Bacterial density data were transformed into natural log before statistical analysis. The means of bacterial load were compared using ANOVA followed by Tukey's post hoc for multiple comparisons. Statistical software SPSS version 20.0 was used to analyze the data with the level of significance at $p<0.05$. For plotting the graphs Microsoft Excel (2010) was used.

2.15 Bioinformatics tools

2.15A FinchTV version 1.4

Geospiza's FinchTV is the popular way to view DNA sequence traces on Linux, Mac OSX, Windows, and Solaris. FinchTV started as the only chromatogram viewer that can display an entire trace in a scalable multi-pane view. And it leads the way with raw data views, BLAST searching and the ability to reverse complement sequences and traces.

2.15B Nucleotide BLAST

The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. *BLAST* can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. In bioinformatics, BLAST for Basic Local Alignment Search Tool is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences (http://blast.ncbi.nlm.nih.gov).

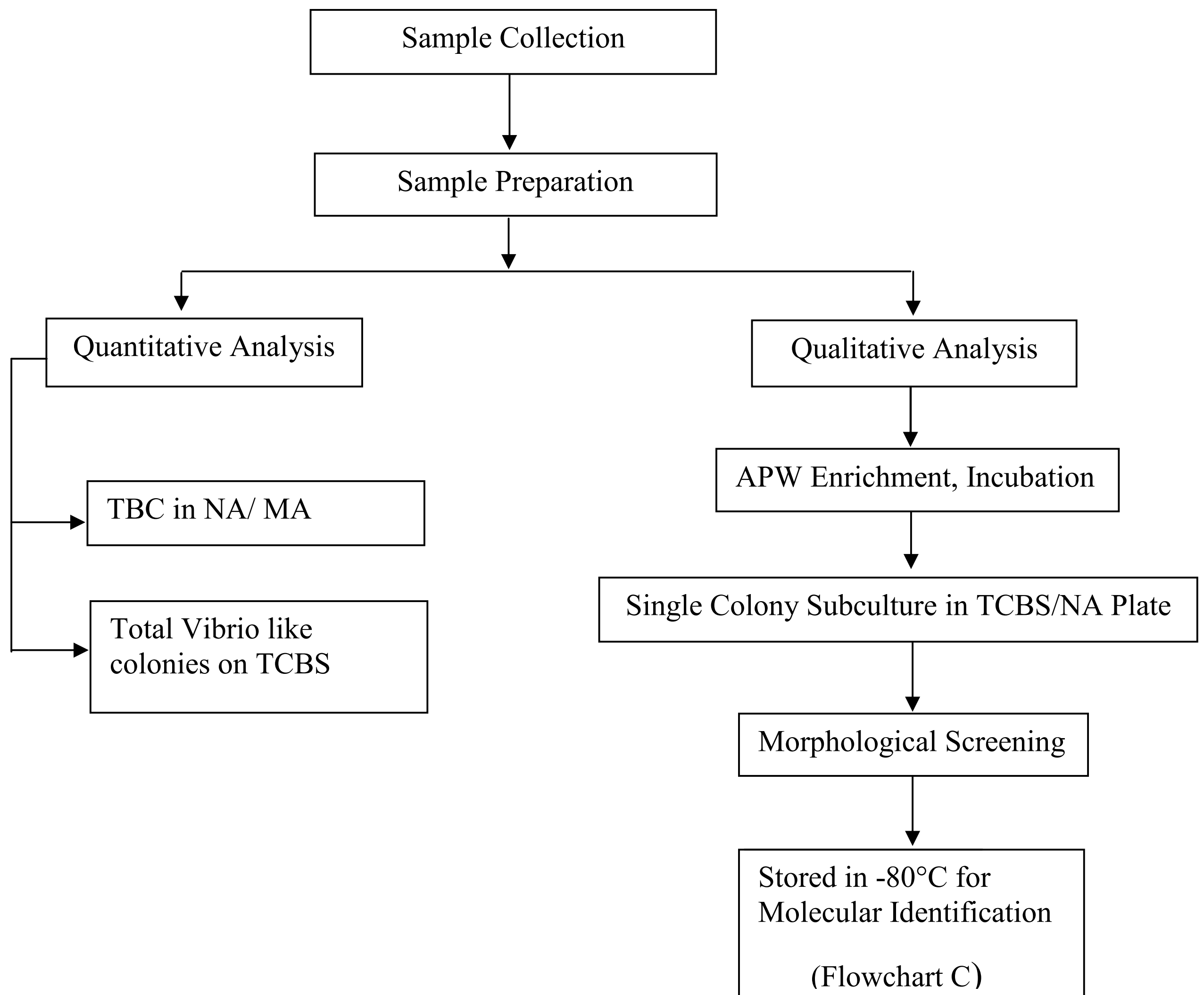
2.15C CLUSTALW

CLUSTAL W improves the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. The sensitivity of the commonly used progressive multiple sequence alignment method has been greatly improved for the alignment of divergent protein sequences. Firstly, individual weights are assigned to each sequence in a partial alignment in order to down-weight near-duplicate sequences and up-weight the most divergent ones. Secondly, amino acid substitution matrices are varied at different alignment stages according to the divergence of the sequences to be aligned. Thirdly, residue-specific gap penalties and locally reduced gap penalties in hydrophilic regions encourage new gaps in potential loop regions rather than regular secondary structure. Fourthly, positions in early alignments where gaps have been opened receive locally reduced gap penalties to encourage the opening up of new gaps at these positions. These modifications are incorporated into a new program, CLUSTAL W which is freely available (Thompson *et al.,* 1994).

2.15D Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software

MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species (Tamura *et al.,* 2013).

2.16 Working protocol of the study



Flowchart B: Enumeration and pure colony isolation

Flowchart C: Molecular identification protocol

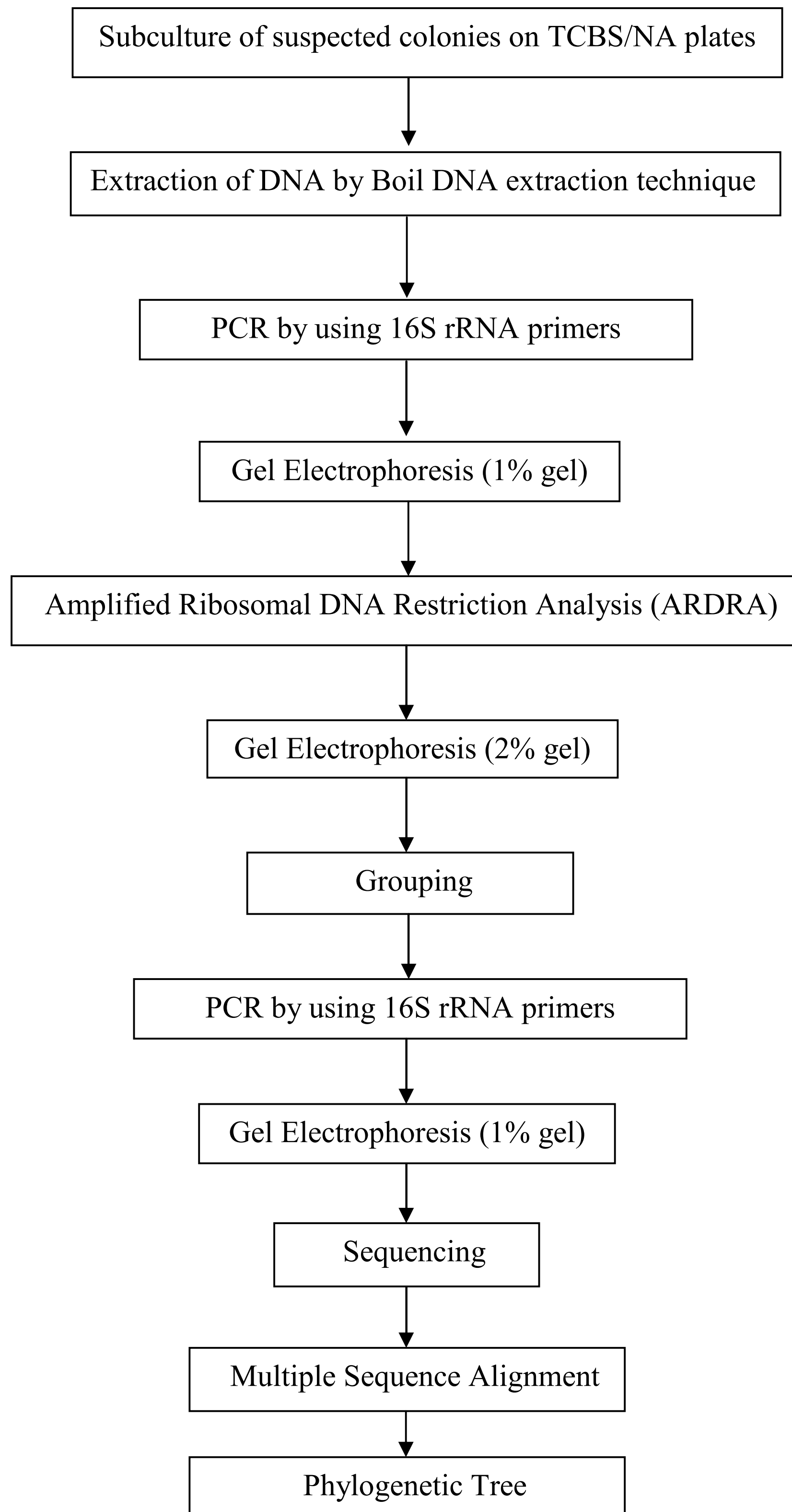


PLATE 1

1A: Sample collection from shrimp hatchery

1B: Sample collection from fish hatchery

1C: Ice box used in sample collection

1D: Shrimp PL sample of 10 days

1E: Artemia nauplii sample

1F: Serial dilution used for bacterial count

PLATE 2

2A: Total Bacterial colonies on NA Plate

2B: Total *Vibrio* like colonies on TCBS Plate

2C: APW Enrichment

2D: Laminar flow

2E: Inoculation in NA

2F: Inoculation in TCBS

Dhaka University Institutional Repository

PLATE 3

 3A: Selection of dissimilar colonies	 3B: Selection of dissimilar colonies
 3C: Pure colony isolate	 3D: Pure colony isolate

3E: Pure colony isolate

Chapter 3

Results

3.1 Quantitative Analysis

3.1.1A Total Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in Nutrient Agar (NA) Plate

The bacterial build up ($2.59 \pm 0.10 \times 10^7$) detected in the water sampled from Artemia hatching tank of Zomzom hatchery, Cox's Bazar is similar to the density observed in the shrimp post larvae (PL) sampled at stage 10 and 12 ($2.37 \pm 0.11 \times 10^7$ and $2.42 \pm 0.10 \times 10^7$ respectively; Figure 3.1A). However, bacterial load determined from the samples of water corresponding to the stages of PL were similar but different from the samples of Artemia tank and PL stages of 10 and 12. But the bacterial build up ($1.38 \pm 0.19 \times 10^7$) found in the PL 8 stage was different from the load sampled from PL tank water and other PL stages.

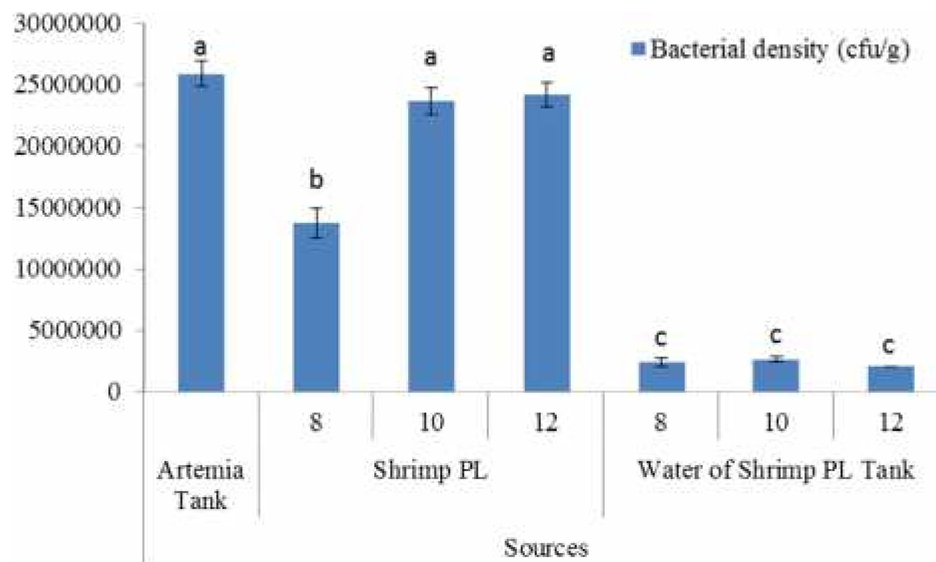


Figure 3.1A Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in Nutrient Agar (NA) Plate. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

3.1.1B Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in Marine Agar (MA) Plate

In MA plate, no significant differences were observed in the bacterial count detected in water sampled from *Artemia* tank and in the PL and corresponding water of PL rearing tank water samples (Figure 3.1B).

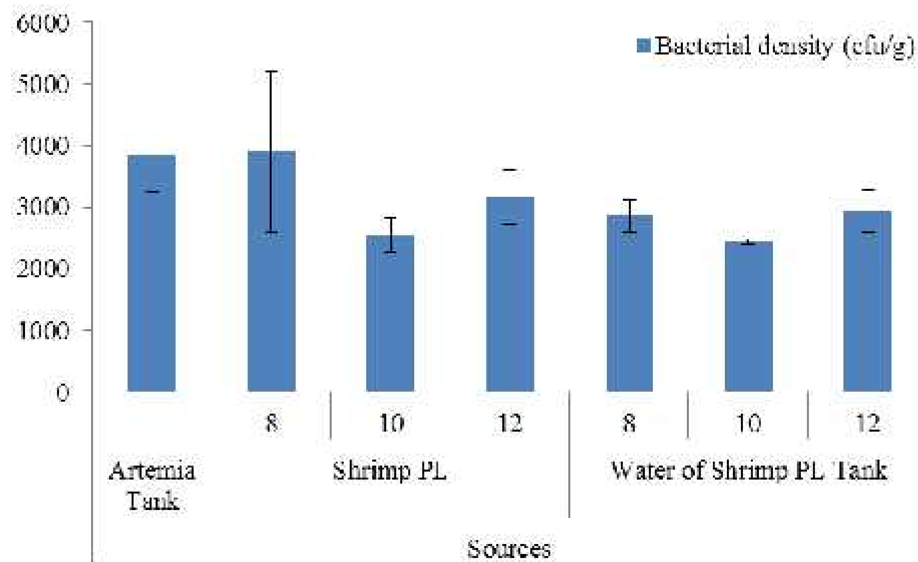


Figure 3.1B Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in Marine Agar (MA) Plate. Bars (mean ± 1 SEM) with no letters indicate no significant difference (ANOVA, HSD; P<0.05).

3.1.1C Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in TCBS Agar (TCBS) Plate

Similar to MA plate, TCBS plates did not result in no significantly different bacterial density in the water sampled from Artemia tank, in the PL and corresponding water sampled from different PL rearing tanks (Figure 3.1C).

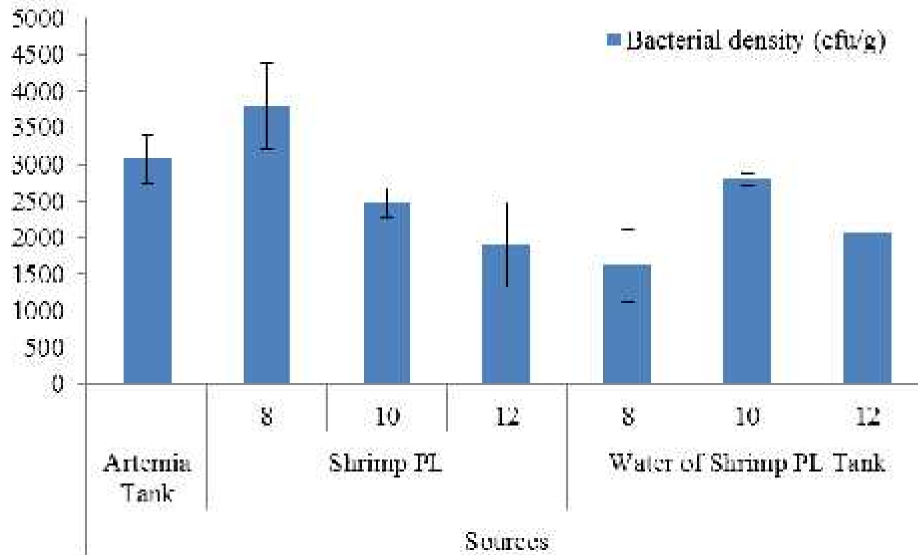


Figure 3.1C Bacterial density (cfu/g) found in Zomzom Shrimp hatchery, Cox's Bazar in TCBS Agar Plate. Bars (mean ± 1 SEM) with no letters denote no significant difference (ANOVA, HSD; P<0.05).

3.1.2A Bacterial density (cfu/g) found in Reliance Tilapia Hatchery, Mymensingh in Nutrient Agar (NA) Plate

In NA plate, the bacterial load ($7.5 \pm 0.11\times10^7$) measured in the water sampled from 25 day old fry rearing pond of tilapia was similar to that of 33 day old fry ($8.6 \pm .66\times10^7$; Figure 3.2A). The bacterial density found in the 25 ($1.6 \pm 0.50\times10^7$), 28 ($3.12 \pm 0.14\times10^7$) and 40 day old fry ($6.46 \pm 1.52\times10^6$) samples were similar but significantly different from the sample of 33 day old fry and the water sample of the pond of 25 day old fry.

3.1.2B Bacterial density (cfu/g) found in Reliance Tilapia Hatchery, Mymensingh in TCBS Plate

In TCBS plate, bacterial abundance detected in the samples across all four age groups were similar (25 day old fry: $4.21 \pm 3.79\times10^3$; 28 day old fry: $4.90 \pm 3.50\times10^3$; 33 day old fry: $1.08 \pm 0.12\times10^3$; 40 day old fry: $7.04 \pm 2.08\times10^3$; Figure 3.2B). No bacterial count was found in the water sampled from 25 day old fry rearing pond.

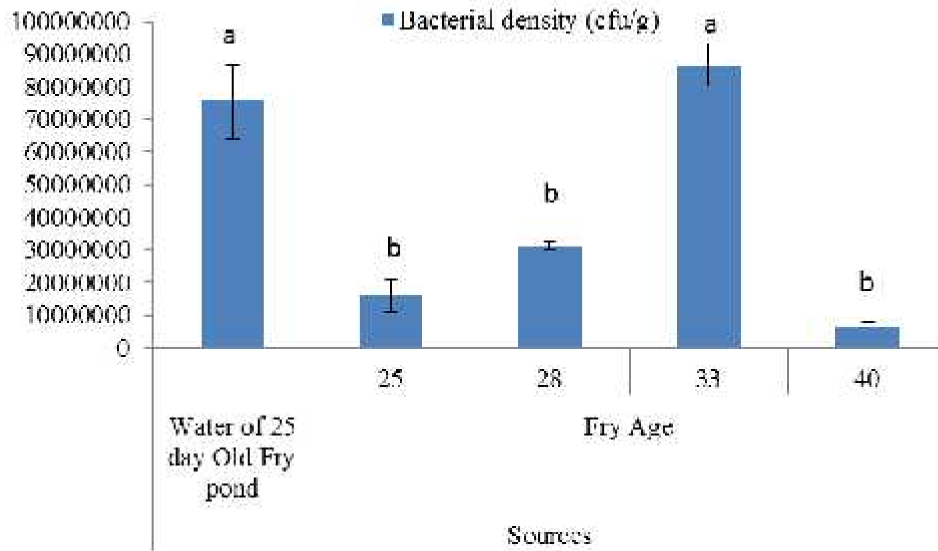


Figure 3.2 A Bacterial density (cfu/g) found in the tilapia fry rearing pond water and in the fries of 25, 28, 33 and 40 days old in Reliance Tilapia Hatchery, Mymensingh in Nutrient Agar (NA) plate. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

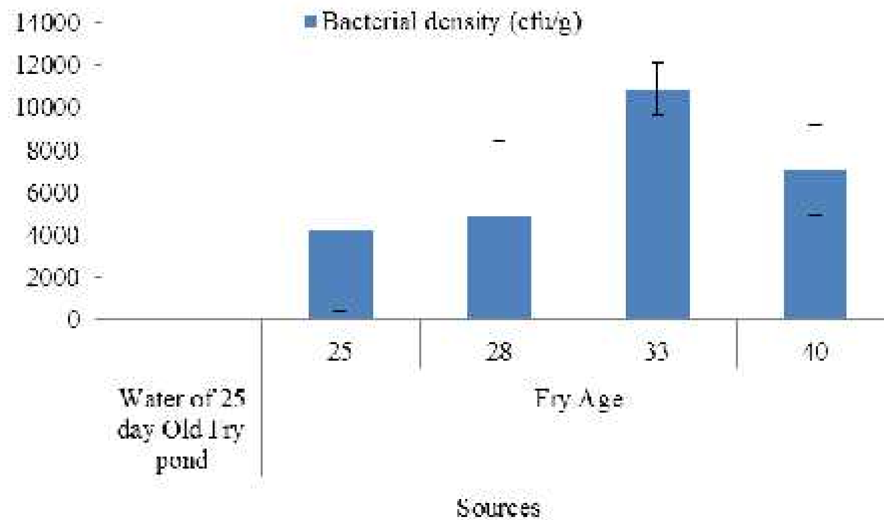


Figure 3.2B Bacterial load (cfu/g) counted in TCBS plate from the 25, 28, 33 and 40 days old fry samples and the water sample of 25 day old fry pond in Reliance Tilapia Hatchery, Mymensingh.

3.1.3A Overall Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in Nutrient Agar (NA) Plate

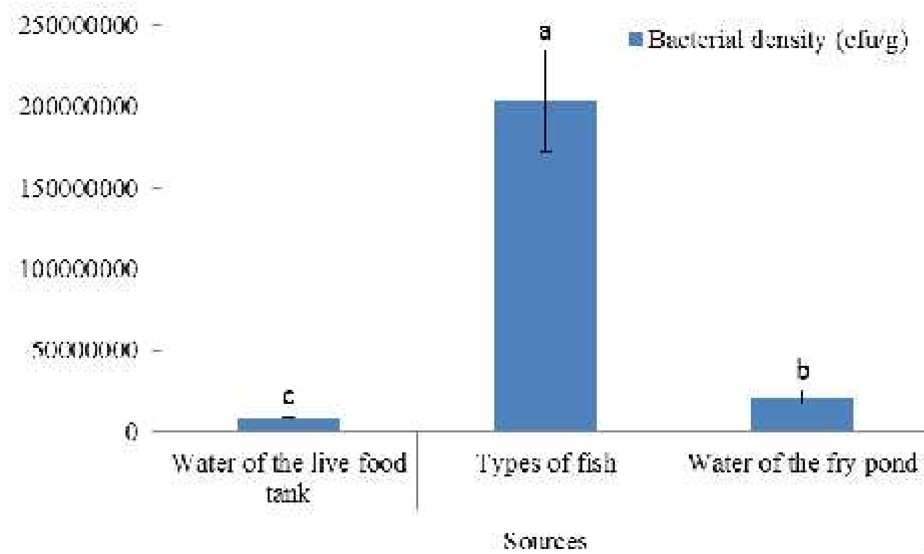


Figure 3.3A Bacterial load (cfu/g) detected in the water sampled from the fry rearing pond of in GM Aquaculture Ltd, Bogra in NA plate. Bars (mean ± 1 SEM) with different letters denote significant difference (ANOVA, HSD; P<0.05).

The overall bacterial build up ($2.03 \pm 0.31\times10^8$) found in the samples of fish fry in NA plate was significantly higher than that of the corresponding rearing pond water ($2.11 \pm 0.459\times10^7$) and the water of the live food rearing tank ($8.43 \pm 0.57\times10^6$; Figure 3.3A).

3.1.3B Overall Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in TCBS

Similar to the overall bacterial density found in NA plate, TCBS plates had 2.3-, and 5.09-folds higher bacterial load ($1.08 \pm 0.25\times10^3$) in the samples of fish fry than in the samples of the corresponding water samples and water samples of the live food rearing tank, respectively ($4.70 \pm 1.67\times10^2$ and $2.12 \pm 0.28\times10^2$; Figure 3.3B).

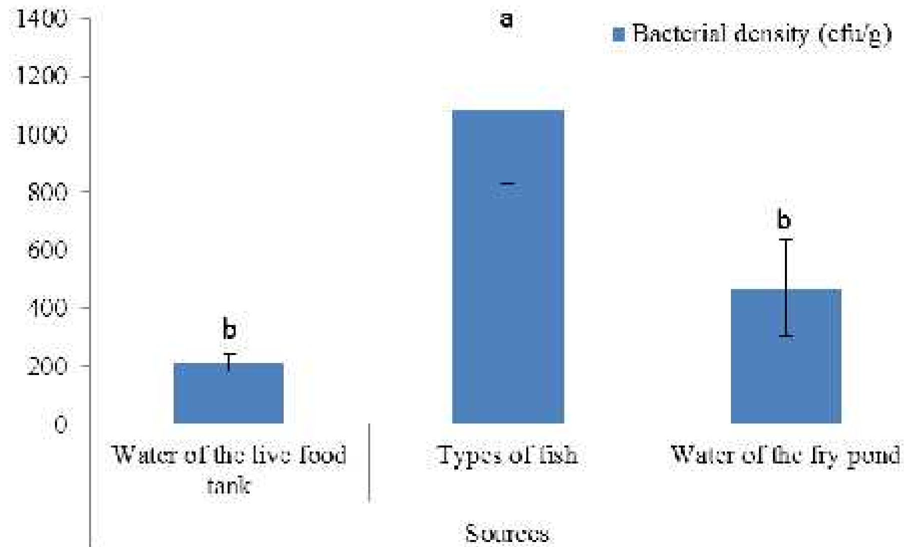


Figure 3.3B Overall bacterial load counted in TCBS plate from the samples of water of the live food tank, types of fish fry and water of the fry rearing pond in GM Aquaculture Ltd, Bogra. Bars (mean ± 1 SEM) with different letters indicate significant difference (ANOVA, HSD; P<0.05).

3.1.3C Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in Nutrient Agar (NA) Plate

In NA plate, the bacterial load found in shing fry ($2.77 \pm 0.11\times10^8$) samples was similar to the density of magur fry ($2.23 \pm 0.03\times10^8$) but significantly higher than in the sample of pangas fry ($1.10 \pm 0.10\times10^8$; Figure 3.3C).

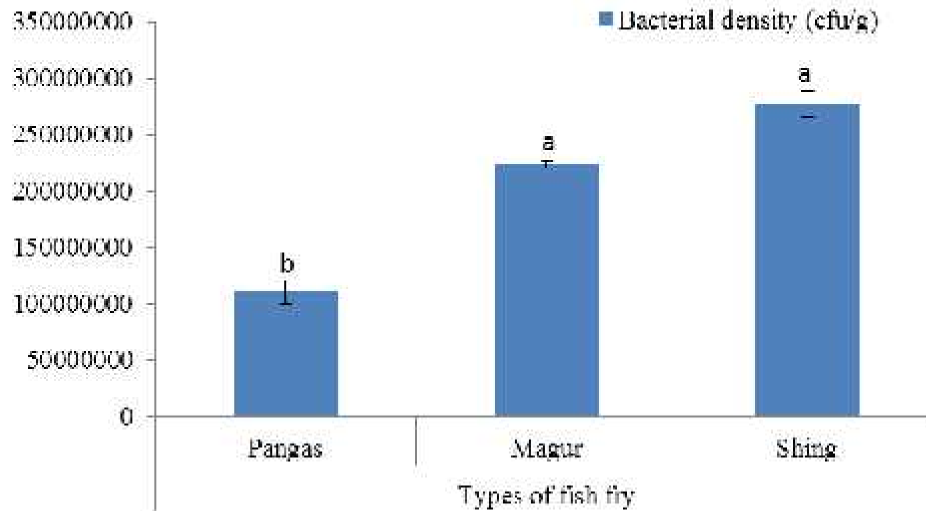


Figure 3.3C Bacterial build up found in the samples of pangas, magur and shing fry sampled from GM Aquaculture Ltd, Bogra in NA plate. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

3.1.3D Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in TCBS Plate

In TCBS plate, while magur fry had the highest density of bacteria ($1.78 \pm 0.06\times10^3$) the lowest density ($4.05 \pm 0.45\times10^2$) was detected in the pangas fry (Figure 3.3D). However, shing fry resulted in the bacterial build up ($1.60 \pm 0.06\times10^3$) that was significantly lower than that of magur fry but higher than did the pangas fry.

3.1.3E Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in NA Plate

In NA plate, while water of pangas and shing fry rearing pond water had similar (pangas: $2.95\times10^7 \pm 0.13\times10^7$; shing: $2.72\times10^7 \pm 0.07\times10^7$) but significantly higher bacterial load that did magur fry rearing pond water ($6.76\times10^6 \pm 0.34\times10^6$; Figure 3.3E).

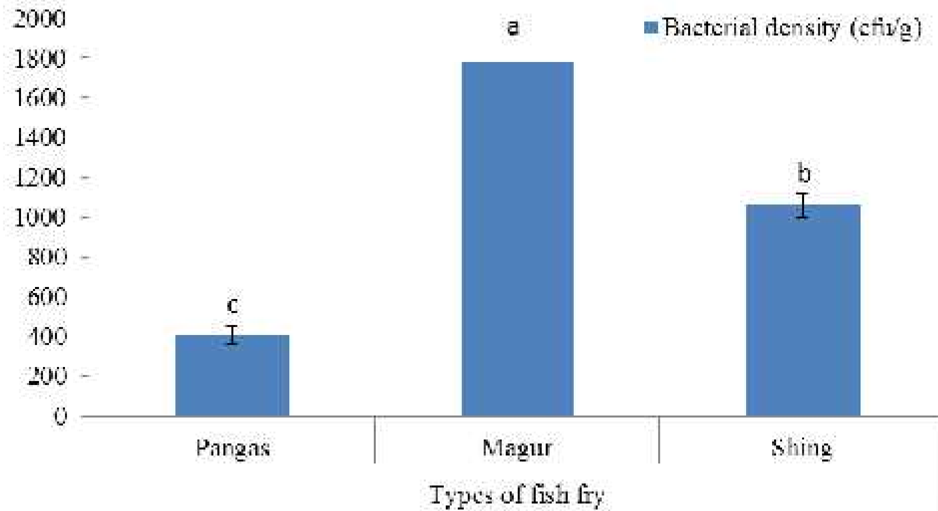


Figure 3.3D Bacterial load detected in TCBS plates in the samples of pangas, magur and shing fry of GM Aquaculture Ltd, Bogra. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

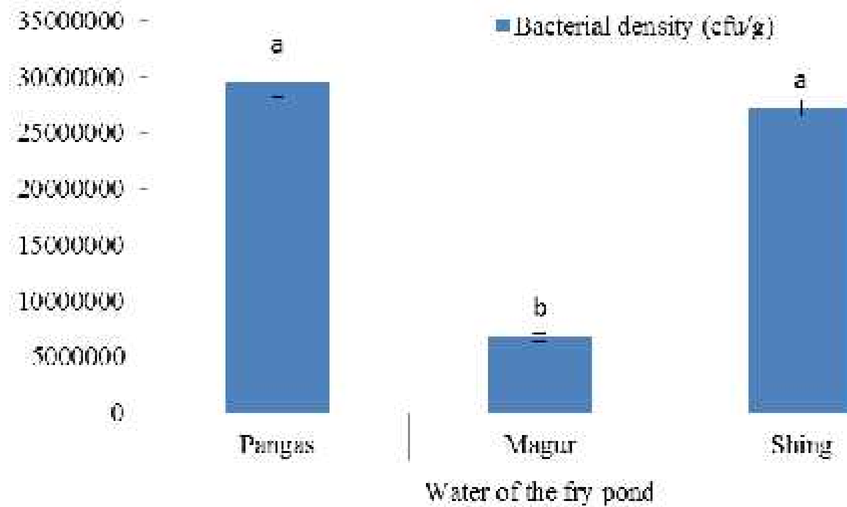


Figure 3.3E Bacterial density counted in NA plate from the water samples of the corresponding pangas, magur and shing fry rearing pond in GM Aquaculture Ltd, Bogra. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

3.1.3F Bacterial density (cfu/g) found in GM Aquaculture Ltd, Bogra in TCBS Plate

In TCBS plate, while the water of magur fry rearing pond had no bacterial load, the shing and pangas fry rearing pond water had 5.10 ± 1.10×10^2 and 9 ± 0.04×10^2, respectively (Figure 3.3F).

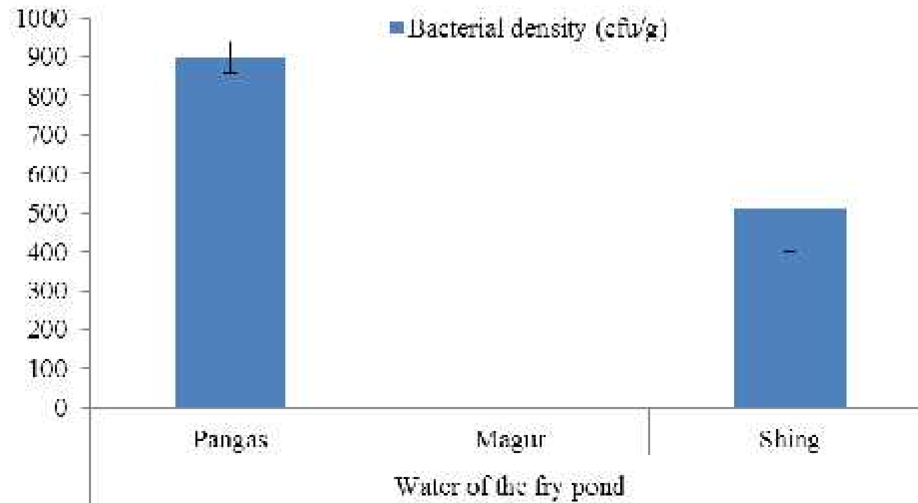


Figure 3.3F Bacterial load (cfu/g) measured in TCBS plates from the samples of water of the corresponding pangas, magur and shing fry rearing pond in GM Aquaculture Ltd, Bogra. Bars (mean ± 1 SEM) with no letters denote no significant difference (ANOVA, HSD; P<0.05).

3.2 Isolation of presumptive *Vibrio* spp.

3.2.1 Thiosulfate-citrate-bile salts-sucrose (TCBS)

Green, yellow and other colored colonies were selected from TCBS cultures and then were streaked on Luria Bertani Agar (LBA; with 3% NaCl supplementation). On TCBS, yellow colonies were presumptively identified as *V. alginolyticus, V. cholerae, V.fluvialis, V. metschnikovii* and *V. furnissii.* Green or blue-green colonies were assumed to be *V. parahaemolyticus, V. vulnificus* and *V. mimicus*. A total of 37 pure colonies were selected by TCBS, from the 30 samples collected from Cox's Bazar and Mymensingh. Morphological dissimilarities of the selected colonies exhibited in TCBS media were presented in the table 3.1.

3.3 Identification of the presumptive *Vibrio* isolates

3.3.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The genomic DNAs purified from the thirty seven (37) suspected *Vibrio* colonies were subjected to a polymerase chain reaction in order to amplify their respective 16S rDNA (Figure 3.4). These produced amplicons of about 1450 bp, which were used as substrate for cleavage by a restriction enzyme, *Alu*I to address their ARDRA pattern. The pattern produced nine different groups for all of the 37 isolates (Figure 3.5). The corresponding bacterial isolates respective to each ARDRA group and the best representative colony from each group is summarized in table 3.2.

Table 3.1: Presumptive 37 *Vibrio* isolates and their colony morphology in TCBS plate that were selected for molecular analysis

Colony ID	Sample ID	Color	Size	Shape	Elevation	Surface
1	C1	Yellow	Medium	Round	Convex	Smooth
2	C3	Blue	Medium	Round	Flat	Smooth
3	C6	Yellow	Small	Round	Convex	Smooth
4	C7	Yellow	Medium	Round	Convex	Smooth
5	C9	Greyish-green	Small	Irregular	Raised	Smooth
6	C10	Green	Small	Round	Flat	Smooth
7	C11	Yellow	Large	Round	Convex	Smooth
8	C12	Yellow	Medium	Round	Convex	Smooth
9	C13	Greenish black	Large	Round	Convex	Smooth
10	C14	Yellow	Large	Round	Convex	Smooth
11	C15	Yellow	Small	Round	Convex	Smooth
12	C16	Yellow	Small	Round	Convex	Smooth
13	C7	Yellow	Small	Round	Raised	Smooth
14	C9	Dark green	Small	Round	Convex	Smooth
15	C10	Green	Medium	Round	Convex	Smooth
16	C2	Yellow	Large	Round	Raised	Smooth
17	C3	Blue	Small	Round	Convex	Smooth
18	C5	Blue	Medium	Round	Convex	Smooth
19	M1	Dark green	Medium	Round	Convex	Smooth
20	M3	Blue green	Medium	Round	Convex	Smooth
21	M4	Greenish	Small	Irregular	Convex	Smooth
22	M5	Blue green	Medium	Round	Convex	Smooth
23	M7	Blue green	Small	Round	Convex	Smooth
24	M7	Greenish	Small	Round	Convex	Smooth
25	M9	Blue green	Medium	Round	Convex	Smooth
26	M12	Blue green	Medium	Round	Flat	Smooth
27	M14	Blue green	Small	Round	Convex	Smooth
28	M1	Blue	Medium	Round	Convex	Smooth
29	M3	Blue green	Small	Round	Convex	Smooth
30	M5	Blue green	Large	Irregular	Flat	Smooth
31	M5	Blue	Small	Round	Convex	Smooth
32	M8	Green	Small	Round	Convex	Smooth
33	M8	Blue green	Medium	Round	Convex	Smooth
34	M11	Blue green	Medium	Round	Convex	Smooth
35	M12	Dark green	Small	Round	Convex	Smooth
36	M13	Blue green	Medium	Round	Convex	Smooth
37	M13	Blue green	Medium	Round	Convex	Smooth

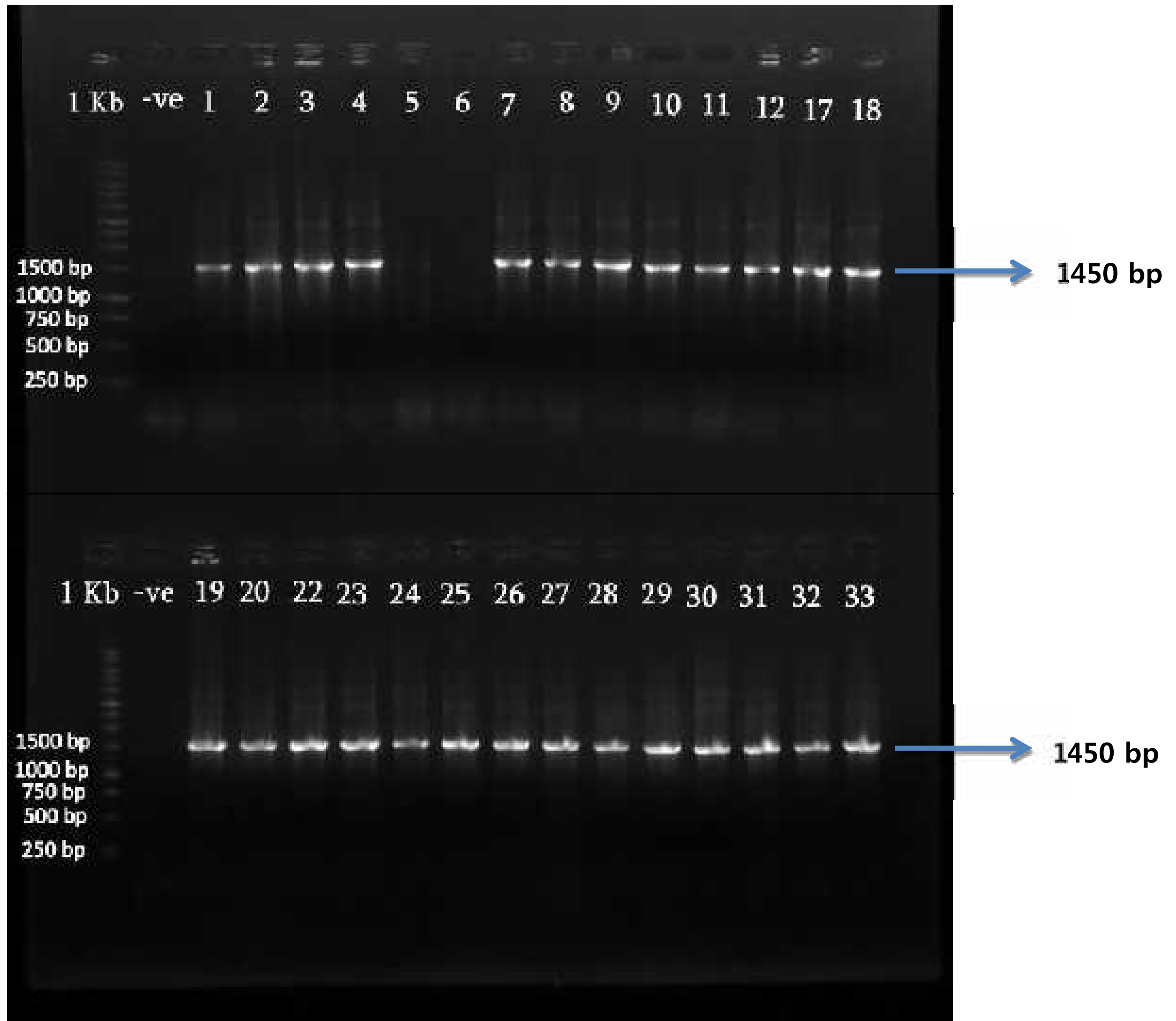


Figure 3.4: Agarose gel (1%) electrophoresis of 16s rDNA gene of 37 isolates. The far left lane is 1 kb DNA ladder, while the next right lane is used as negative control.

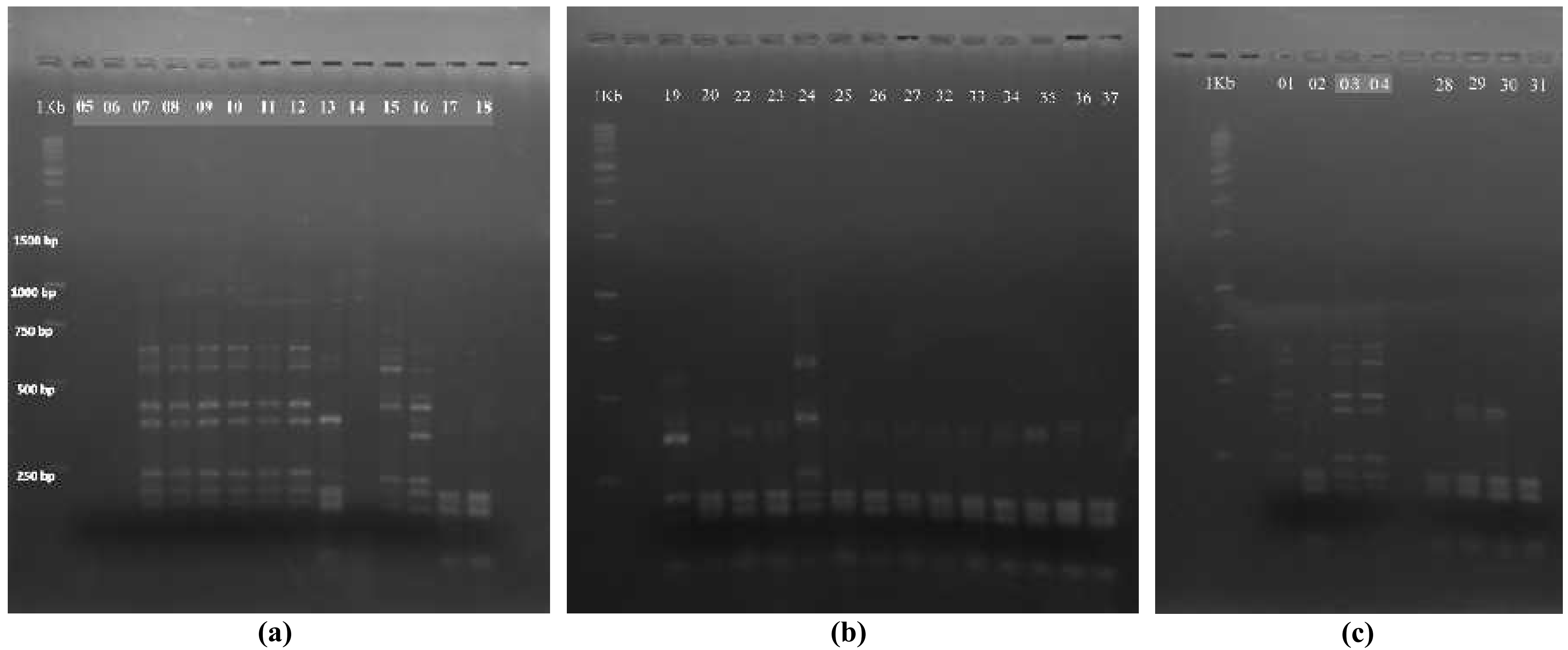


Figure 3.5: ARDRA pattern analysis. Restriction digestion of bacterial isolates using *Alu*I enzyme, the left most lane in all 3 figures indicates 1kb DNA marker.

Table 3.2: The ARDRA pattern of the isolates

ARDRA Group	Representative Colony ID	Colony ID
ARH 1	7	1 ,3, 4, 7, 8, 10, 11, 12
ARH 2	10	10
ARH 3	18	2, 18, 17, 28, 31
ARH 4	13	13
ARH 5	15	15
ARH 6	16	16
ARH 7	19	19
ARH 8	24	24
ARH 9	26	26, 25, 27, 32, 33, 34, 35, 36, 37, 20, 22, 23,29,30

The nine ARDRA groups were then subjected to 16S rRNA PCR again, and from 1% agarose gel electrophoresis (Figure 3.6) it is clear that there are eight groups of isolates. The ARDRA group ARH 2 is merged into the group ARH 1.

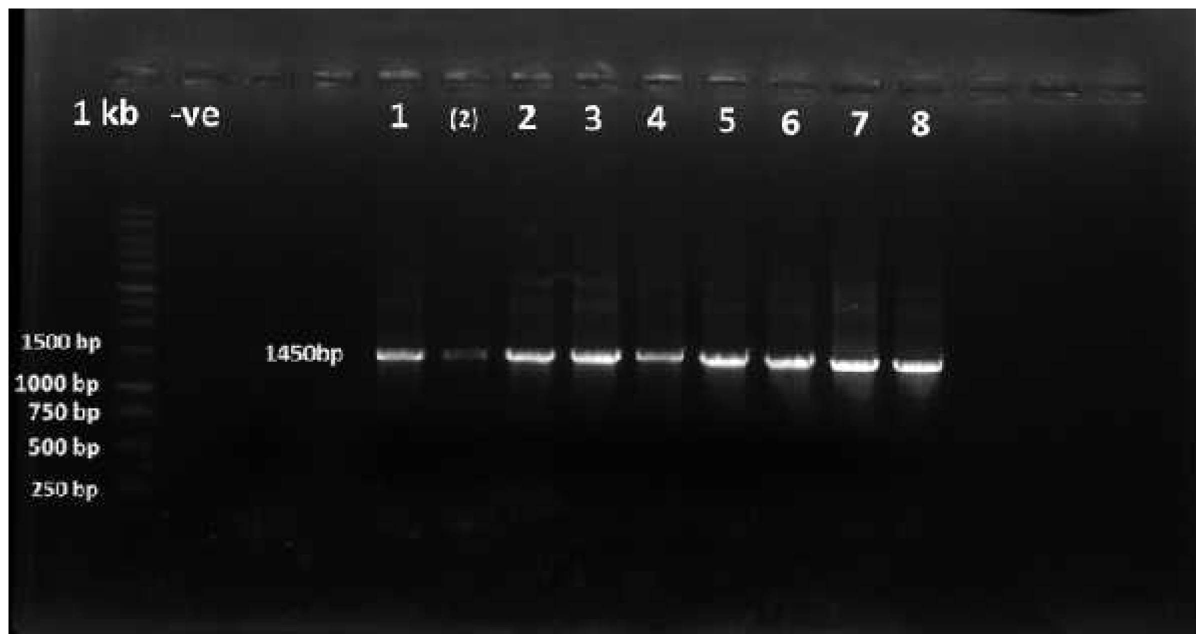


Figure 3.6: Agarose gel (1%) electrophoresis of 16s rDNA gene of 9 ARDRA groups. The far left lane is 1 kb DNA ladder, while the next lane is used as negative control.

The final concentration and purity of the eight groups measured before 16S rRNA sequencing with their corresponding coloy ID and sample ID are summarised in the following table 3.3.

Table 3.3: Summary of the eight representative isolates used for sequencing

Group	Concentration (ng/µL)	Purity	Colony ID	Sample ID
ARH 1	68.4	1.97	1	C1
			3	C6
			4	C7
			7	C11
			8	C12
			10	C14
			11	C15
			12	C16
ARH 2	114.0	1.94	2	C3
			17	C11
			18	C5
			28	M1
			31	M5
ARH 3	113.1	1.94	13	C7
ARH 4	80.5	1.96	15	C 10
ARH 5	77.0	1.95	16	C 2
ARH 6	111.6	1.95	19	M1, M2
ARH 7	106.8	1.95	24	M7, M8, M9
ARH 8	114.5	1.94	20	M3
			22	M5
			23	M7
			25	M9
			26	M12
			27	M14
			29	M3
			30	M5
			32	M8
			33	M8
			34	M11, M12
			35	M12
			36	M13
			37	M13, M14

3.3.2 16S rRNA sequence based identification

The identification of the 16S rDNA gene sequences of eight representative isolates of the eight groups (ARH 1, ARH 2, ARH 3, ARH 4, ARH 5, ARH 6, ARH 7 and ARH 8) through nucleotide BLAST of NCBI is summarised in table 3.4.

From table 3.4, it is clear that the sequences of all the groups of isolates were matched with the strains presented in their respective following column. The table also provides information about scoring (maximum score, total score, query cover, E value and percentage of identification) of the identified sequences with NCBI Gene Bank Accession number of the matched sequences.

As group ARH 1 is identified as *Vibrio alginolyticus,* all the isolates of this group can also be identified as *Vibrio alginolyticus.* Similar conclusions can also be drawn with the other groups about their corresponding isolates.

In table 3.5, a total summary of identification of all the isolates has shown with their corresponding sample name that were collected from hatchery environments of Bangladesh. Three of eight of our representative isolates that are ARH 1, ARH 4 and ARH 5 were identified as *Vibrio alginolyticus, Vibrio vulnificus* and *Vibrio cholerae*, respectively. The table shows that the samples in which these *Vibrio* species found were exclusively collected from the coastal environment of Cox's Bazar. Among these three groups, ARH 1, which was identified as *Vibrio alginolyticus,* represents the highest number of isolates including samples.

Overall, of the total eight representative groups, 3 species were under the genus *Vibrio*, 3 of the genus *Aeromonas*, one *Edwardsiella hoshinae* and one *Bacillus methylotrophicus* species as identified.

Table 3.4: 16s rRNA sequence (1320 bp) based identification of representative isolates from hatchery environment

Group	Identified Strain	Max score	Total score	Query cover	E value	Identities	Gene Bank Accession No.
ARH 1	*Vibrio alginolyticus* strain PE2 16S rRNA	2464	2464	100%	0	100%	KT036618.1
ARH 2	*Aeromonas veronii* strain WX153415 16S rRNA	2590	2590	100%	0	100%	KT964297.1
ARH 3	*Aeromonas hydrophila* strain A-X4 16S rRNA	2577	2577	100%	0	99%	KJ806490.1
ARH 4	*Vibrio vulnificus* strain FORC_009 chromosome 2	2514	2514	100%	0	99%	CP009985.1
ARH 5	*Vibrio cholerae* strain BB31	1908	1908	100%	0	100%	KF446244.1
ARH 6	*Edwardsiella hoshinae* strain ATCC 35051 16S rRNA	2591	2591	99%	0	99%	KM676416.1
ARH 7	*Bacillus methylotrophicus* strain LD34 16S rRNA	2608	2608	100%	0	100%	KR855694.1
ARH 8	*Aeromonas veronii* strain K11 16S rRNA	2013	2013	100%	0	100%	KU041801.1

Table 3.5: Summary of molecular identification of the samples drawn from fish and shrimp hatchery environments of Bangladesh

Group	Identified sequence	Colony ID	Sample ID	Sample
ARH 1	***Vibrio alginolyticus* strain PE2 16S rRNA**	1	C1	Artemia from Tank 1
		3	C6	Water from PL(10) tank
		4	C7	Shrimp PL of 12 days
		7	C11	Shrimp PL of 8 days
		8	C12	Water from PL(8) tank
		10	C14	Water from PL(12) tank
		11	C15	Shrimp PL of 10 days
		12	C16	Water from PL(10) Tank
ARH 2	*Aeromonas veronii* strain WX153415 16S rRNA	2	C3	Shrimp PL of 10 days
		17	C11	Shrimp PL of 8 days
		18	C5	Shrimp PL of 10 days
		28	M1	Tilapia fry, 40 days,
		31	M5	Tilapia fry, 25 days
ARH 3	*Aeromonas hydrophila* strain A-X4 16S rRNA	13	C7	Shrimp PL of 12 days
ARH 4	***Vibrio vulnificus* strain FORC_009 chromosome 2**	15	C10	Artemia from Tank 2
ARH 5	***Vibrio cholerae* strain BB31**	16	C2	Artemia from Tank 1
ARH 6	*Edwardsiella hoshinae* strain ATCC 35051 16S rRNA	19	M1,M2	Tilapia fry, 40 days
ARH 7	*Bacillus methylotrophicus* strain LD34 16S rRNA	24	M7, M8, M9	Tilapia fry, 28days
ARH 8	*Aeromonas veronii* strain K11 16S rRNA	20	M3	Tilapia fry, 40 days
		22	M5	Tilapia fry, 25 days
		23	M7	Tilapia fry, 28days
		25	M9	Tilapia fry, 28days
		26	M12	Tilapia fry, 33days
		27	M14	Water of 25 day old tilapia fry pond
		29	M3	Tilapia fry, 40 days
		30	M5	Tilapia fry, 25 days
		32	M8	Tilapia fry, 28days
		33	M8	Tilapia fry, 28days
		34	M11, M12	Tilapia fry, 33days
		35	M12	Tilapia fry, 33days
		36	M13	Water of 25 day old tilapia fry pond

3.3.3 Multiple Sequence Alignment

In figure 3.7 while compared the observed sequences of the groups ARH 1, ARH 4 and ARH 5 denotes 76 polymorphic sites among them. Therefore, the dissimilarity was 5.7% (76/1320=0.057). ARH 1 was closely related to ARH 4 while ARH 4 to ARH 5. ARH 1 and ARH 5 were found poorly related to each other than they were with the ARH 4.

Similarly, in figure 3.8 while compared, 171 polymorphic positions were found among the groups of ARH 1, ARH 2, ARH 3, ARH 4, ARH 5 and ARH 8. Thus the variation in the basepair was 12.9% (171/1320=0.1295). Alignment clearly showed that group ARH 1, ARH 4 and ARH 5 were closely related to each other than they were with the group of ARH 2, ARH 3 and ARH 8. Taxonomic relations among the group of the first lot (ARH 1, ARH 4 and ARH 5) was best seen in ARH 3, ARH 2 and ARH 8, respectively.

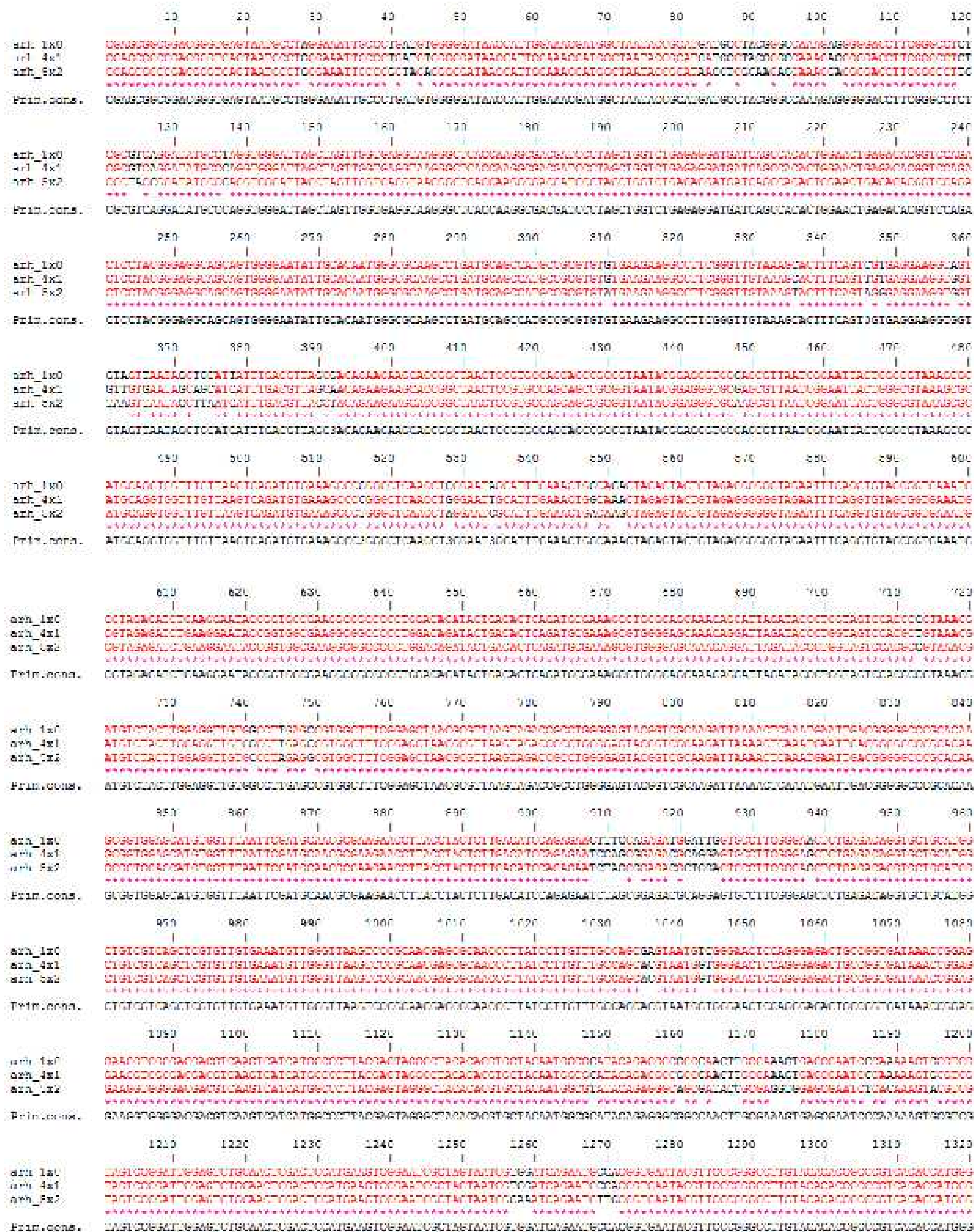


Figure 3.7: Multiple sequence alignment of 16S rRNA gene fragments of the closely related group ARH 1, ARH 4 and ARH 5 where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 120)

Figure 3.8A: Multiple sequence alignment of 16S rRNA gene fragments of the group ARH 1, ARH 2, ARH 3, ARH 4, ARH 5 and ARH 8 where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 120)

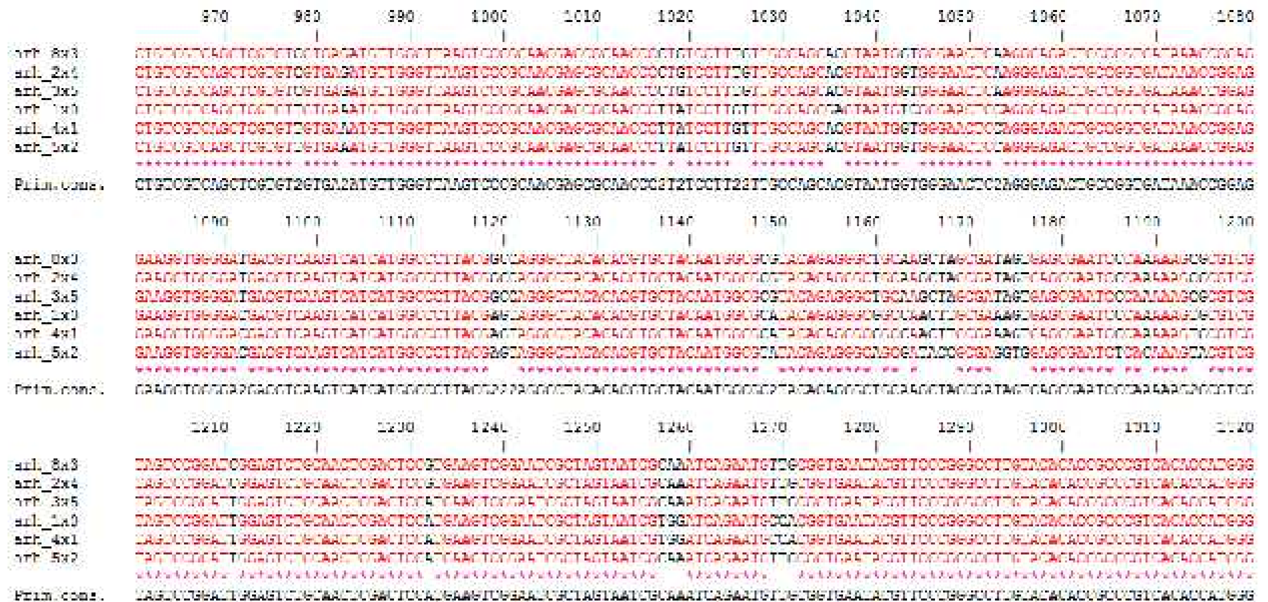


Figure 3.8B: Multiple sequence alignment of 16S rRNA gene fragments of the group ARH 1, ARH 2, ARH 3, ARH 4, ARH 5 and ARH 8 where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 120)

3.3.4 Phylogenetic Analysis

Phylogenetic analysis (Figure 3.9) based on the partial 16S rDNA gene sequences of the representative eight isolates using neighbor-joining confirmed the taxonomic position of the isolate ARH 1, ARH 4 and ARH 5 of the genus *Vibrio,* and allocated ARH 1 to the strain *Vibrio alginolyticus* PE2, ARH 4 to the strain *Vibrio vulnificus* FORC_009 and ARH 5 to the strain *Vibrio cholerae* BB31. From the tree it is also clear that ARH 1 is closely related to the strain of *Vibrio parahaemolyticus* CECT 611, *V. rotiferianus* LPD 1-1-86, *V. campbellii* R 1311, *V. ordalii* NCMB 2168 and *V. vulnificus* FORC 009, whereas the closeness of taxonomic relation with ARH 4 is just opposite to the direction of this series. The phylogenetic tree confirms the taxonomic position of ARH 3, ARH 2 and ARH 8 in the genus *Aeromonas* supporting their similarity with the strain *A. hydrophilla* A-X4, *A. veronii* WX153415 and *A. veronii* K11 respectively. The taxonomic relation of ARH 6, which is allocated to *Edwardsiella hoshinae* ATCC 35051, is closer with the genus *Aeromonas* than with *Vibrio* spp.

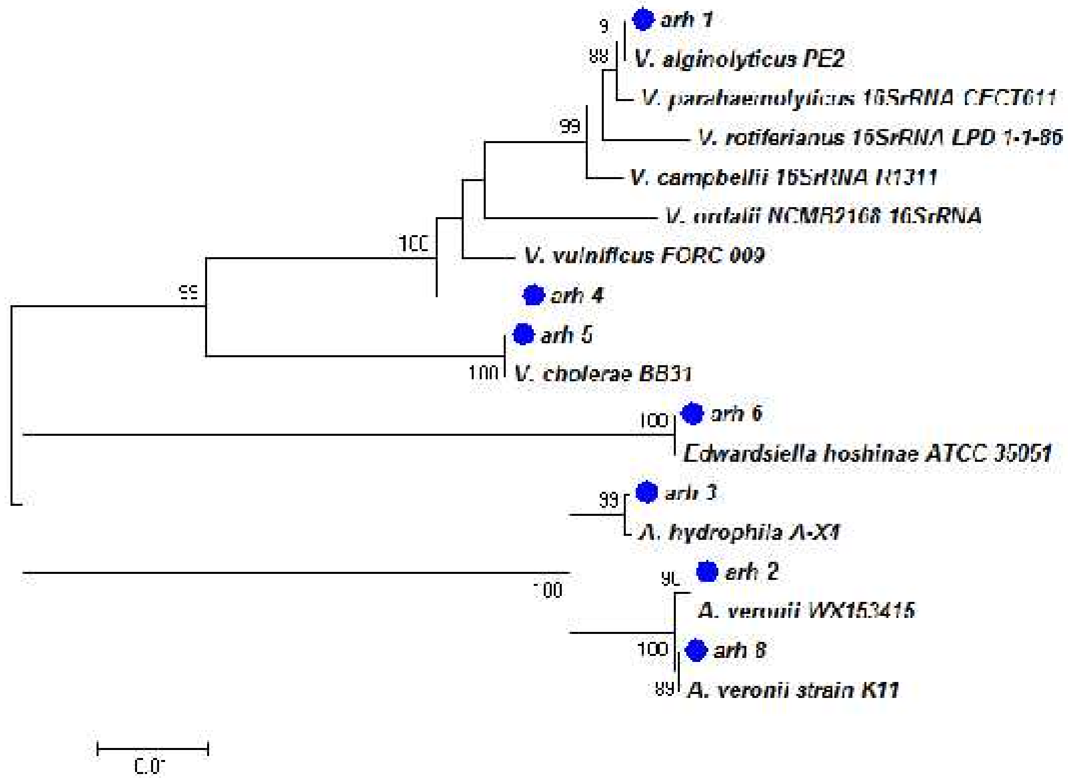


Figure 3.9: The neighbour-joining (NJ) phylogenetic tree based on partial 16S rRNA gene sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method. Numbers in tree are bootstrap values. Blue circle indicates position of the studied strains.

Phylogeny Test

Test of Phylogeny: Bootstrap method

No. of Bootstrap Replications: 1000

Substitutions Type : Nucleotide

Model/Method : Maximum Composite Likelihood

Substitutions to Include: -d: Transitions + Transversions

Rates among Sites: Uniform rates

Pattern among Lineages: Same (Homogeneous)

No. of Sites: 1320

No Of Bootstrap Reps = 1000

Chapter 4

Discussion

Bacterial flora on fish reflects the aquatic environment which affects the quality and storage life of fishery products (Shewan, 1976). It has been repeatedly suggested that the bacterial flora of fish might reflect the bacteriological conditions of the water and a potential indicator of pollution. Therefore, to understand the vulnerability and quality of the hatchery environments and to detect the prevalence of *Vibrio* spp., both total bacterial count and total presumptive *Vibrio* count was taken for the samples using NA and TCBS media, where the later is selective for *Vibrio* like species. MA is used only for the shrimp hatchery samples to understand the overall density of the marine micro flora.

Similar bacterial density between Artemia tank and shrimp PL as observed in this study in NA plate could be due to the use of Artemia nauplii and shrimp PL as the host organisms. Lower density of total bacteria in the water of PL rearing tank in comparison with the PL, supports the findings of Rao (2013) and denotes the absence of host organisms. Progressively higher density of total bacterial abundance in the shrimp PL of stages from 8 to 12 could be due to size variation.

Only few thousands (<5000 cfu/g) bacterial abundance in MA plate across all samples of Artemia tank, shrimp PL and the water of the PL rearing tank could have resulted since the growth was observed in marine selective media. Therefore, the growth was similar across all samples.

In TCBS plate, bacterial density ranged between 1620 and 3800 cfu/g which also indicates selective growth of *Vibrio* spp. But shrimp PL had greater mean loads of presumptive *Vibrio* than in their surrounding water body which was similar with the previous reports (Otta *et al.,* 2001).

Very high bacterial abundance ($7.55 \pm 0.11\times10^7$) as found in the water of the 25 day old tilapia fry rearing pond could have resulted due to anthropogenic contamination. Interestingly, 40 day old tilapia fry had 10 times lower bacterial build up than in the 33 day old tilapia fry. The reason responsible behind this variation in the total count of bacteria in

NA plate is unknown. Typically smaller fish should have lower bacterial load compared to the bigger ones.

In TCBS plate, no growth of any bacteria denotes absence of *Vibrio* spp. in 25 day old tilapia fry rearing pond water. This absence of *Vibrio* in the fry rearing pond water indicates no contamination from anthropogenic sources as well as denotes the quality level of the fry feed. However, tilapia fry aged from 25-40 day old had similar *Vibrio* growth that indicates the possibility of later contamination from unknown sources. Even environmental parameters such as temperature, salinity, pH and dissolved oxygen play a foremost part in the distribution of bacteria (Palaniappan *et al.,* 1982; Parvez *et al.,* 2015).

Nearly 10 and 20 times higher total bacterial abundance in the fry of pangas, magur and shing compared to that of live food rearing tank water and the water of fry rearing ponds in NA plate could also be responsible for the established fact that fish body carries higher microbial organisms than that of surrounding water body. This may be a result of the high organic load in the incoming water (Otta *et al.,* 2001). The same reasons could also be blamed for the presumptive *Vibrio* spp. growth in TCBS plates of the corresponding samples.

Particularly the reason of significant differences in total bacterial load in NA plate between the pangas fry and the near similar magur and shing fry could be the discrepancy in their hardiness. The hardier the fish species the more bacterial load it may contain. The statistical significant difference in the amount of *Vibrio* like bacteria of the pangas, magur and shing fry samples in TCBS plate also advocates for the previously stated reason.

The bacterial load in NA plate of the water samples of magur fry rearing pond is significantly different from the water samples pangas and shing fry rearing pond. Water of magur fry rearing pond has lowest both TBC and TVC in NA and TCBS plates, respectively. The reason behind this variation perhaps lies on the treatments applied and the quality of the water of the corresponding fish fry rearing ponds. However, the mean total bacterial density in the fry rearing ponds exceeded the reported range of Otta *et al.,* 2001; while the mean total presumptive *Vibrio* count was a bit lower than their recommended

values. Better water management systems adopted by hatcheries might play important role in this respect (Surendran *et al.,* 2013).

The dominance of *Vibrio* species in hatchery environments is well established by the works of Groumellec *et al.* (1995) and Tanasomwang and Ruangpan (1995). After inoculation and purification in TCBS, the highly selective media for *Vibrio* spp., presumptive *Vibro* colonies were screened from the samples based on their morphological dissimilarities so that as many variations as possible could be taken for further confirmation through molecular approaches.

Several studies have been conducted to compare the usefulness of 16S rDNA sequencing with conventional or commercial methods for the identification of various groups of medically important bacteria (Poretsky *et al.*, 2014; Clarridge, 2004; Matsumoto *et al.* 2013). In general, 16S rDNA sequencing results in a higher percentage of species identification than conventional or commercial methods. The success rate of species identification by 16S rDNA sequencing ranged from 62 to 92%, depending on the group of bacteria and the criteria used for species definition (Hall *et al.,* 2003; Bosshard *et al.,* 2003, 2004; Song *et al.,* 2005; Heikens *et al.,* 2005). Unlike phenotypic identification, which can be affected by the presence or absence of non-housekeeping genes or by variability in expression of characters, 16S rDNA sequencing provides accurate identification of isolates with atypical phenotypic characteristics.

The reasons behind the use of 16Sr RNA in bacterial identification in this study are numerous. The gene has a suitable length of about 1550 bp which is large enough, with sufficient interspecific polymorphisms to provide distinguishing and statistically valid measurements. Besides, 16S rRNA gene is universally distributed in all bacteria (Woese *et al.,* 1987, 1995), thus relationships can be measured among them. The whole sequence of 16S rRNA is highly conserved (greengenes.lbl.gov) and functionally constant through evolutionary history. They have 'hyper variable regions' (Clarridge, 2004) - highly varied between other species but completely similar in the same species. 16S rRNA shows unique signature sequence in same species.

The ARDRA approach was practiced in this study for the purpose of accurate grouping among the morphologically dissimilar isolates. Grouping of isolates reduces the time and

cost of molecular identification. ARDRA has been found to be a useful tool for identification of bacterial isolates in a clinical routine laboratory, because of its speed–compared to phenotypic identification, its reliability, practical applicability, flexibility and the possibility to identify most bacteria together with and at an affordable price (De Baere *et al.,* 2002). Vaneechoutte *et al*. (1995) have shown that ARDRA method is technically less demanding than other molecular biology approaches, secondly, it allows for identification purpose within one day, when starting from a pure culture. ARDRA was recently reported to be a rapid and efficient method of bacteria identification even at the species level (Olivares-Fuster *et al.,* 2007; Schlegel *et al.,* 2003; Spergser *et al.,* 2007; Mendoza-Espinoza *et al.,* 2008; Shkoporov *et al.,* 2008; Najjari *et al.,* 2008). For these reasons, it was reasonable and interesting to use ARDRA in this study of community analysis of *Vibrio* species.

*Alu*I, the restriction enzyme used in digestion in ARDRA, was best recommended by Szczerba *et al.,* 2009 and Vaneechoutte *et al.,* 1995. Szczerba (2009) found the highest number of the common fragments in the restriction profiles that had digested with *Alu*I. The restriction analysis of this study detected 9 different groups of isolates one of which, ARH 2, was later merged with group ARH 1 after the final confirmatory gel electrophoresis.

In the sequenced 8 representative isolates, 3 *Vibrio* species are found among which the maximum number of isolates has been identified as *Vibrio alginolyticus* (ARH 1). The samples, in which these 3 *Vibrio* species are found, were exclusively collected from coastal environment. This finding is in agreement with the observation that occurrence *Vibrio*s is plentiful in marine and coastal environment than in freshwater (Gomez-Gill *et al.,* 2014). However, in support of the current study, it might be stated that the dominance of *V. alginolyticus* in the shrimp hatchery samples was also found by Rao *et al.* (2013). Bhaskar *et al.* (1998) and Felix (2000) also described *V. alginolyticus* as the most common vibrio species in the shrimp hatchery environments.

V. alginolyticus is a pathogenic bacterium for human and many of the infections occur when seawater got into contact with open wounds or other trauma (Rubin and Tilton 1975). This species has been implicated as the causal agent of vibriosis or gas gut disease of many marine aquaria fishes (Stoskopf 1993). *V. alginolyticus* is also described as a pathogen for

shrimp farming (Jayasree *et al.,* 2006). The interaction of virus and *V. alginolyticus* in the earlier stage of virus disease of *P. chinensis* showed that insidious infection of vibrio is advantageous to the infection of virus (Ding *et al.,* 2000). The presence of this bacterium also indicates its resistance against the treatments applied on the hatchery water.

The representative strain ARH 4 is identified as *Vibrio vulnificus* which is an established human pathogen (Gomez-Gill *et al.,* 2014; Amaro and Biosca 1996) and responsible for many food borne dieases (Blake *et al.,* 1979; Shapiro *et al.,* 1998). Similar to the findings of this observation Rao *et al*. (2013) detected the bacteria only in shrimp hatchery samples. The source of this strain was water from the artemia tank that was accused as a source of *V. harveyi* in the works of Vaseeharan & Ramasamy (2003).

Vibrio cholerae was found in the isolate ARH 5 which was also from the same source as *V. vulnificus.* The presence of this bacterium denotes fecal contamination in the water that might has happened due to poor sanitation or uncleanliness of the hatchery laborers or operators. This bacterium is a well-recognized human pathogen associated with cholera disease (Gomez-Gill *et al.,* 2014). However, only 2 of 18 bacterial isolates collected from Cox's Bazar samples indicates better quality of the hatchery water.

Interestingly, good amount of isolates showed similarity with *Aeromonas* species though TCBS (selective for Vibrio spp.) media has been used for isolation. This might be the fact that *Aeromonas* spp. has partial inhibitory growth in TCBS agar media (Public Health England, 2015).

Multiple sequence alignment has been done for the *Vibrio* and *Aeromonas* species as they are more or less closely related to each other than they are with the other two found strains. This approach has been used to identify the polymorphic sites in the base pair of the subjected strains. The position of bacteria in the alignment represents their similarity that's why all 3 *Aeromonas* species clustered first and then the *Vibrio* species earn their places. However, this positioning is based on the amount of polymorphic sites in the isolates.

The constructed phylogenetic tree, involved a total of 18 (7 of our isolates + 11 downloaded from NCBI GenBank) nucleotide sequences, supports the output of the multiple sequence

alignment. To robust the positioning of isolates and to ascertain about their taxonomic position 4 more related reference strains were downloaded from NCBI GenBank. The tree was constructed using the Neighbor-Joining method to understand the inference about evolutionary history. The optimal tree with the sum of branch length = 0.24803246 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. There are a total of 1320 positions in the final dataset, which is in compliance with the recommended ideal guidelines (for less than 1% ambiguities) for use of 16S rRNA gene sequencing for microbial identification. Evolutionary analyses are conducted in MEGA6 following the instruction of Tamura *et al.* (2013).

Chapter 5

Conclusion and Recommendations

5.1 Conclusion

This study describes the presence of *Vibrio* species in the sampled hatcheries and helps to comment about the quality of the hatchery environment through counting their microbial loads. The quantitative analysis of total bacterial count and total presumptive *Vibrio* count also reveals the efficiency of the treatments applied to the hatchery water.

The presence of pathogenic *V. alginolyticus, V. vulnificus* and *V. cholerae* in the Zomzom hatchery (shrimp hatchery) indicates a possibility of future outbreak of vibriosis and other diseases.

The findings of this study also questions about the way of using livefood (artemia) in the respective shrimp hatchery as all of the 3 pathogenic *Vibrio* species were found in the samples collected from the artemia tank.

The results of this study also denote the dominance of *V. alginolyticus* in the shrimp hatchery environment. However, the absence of *Vibrio* species in the freshwater fish hatchery is not unquestionable and demands further research on this aspect.

Indeed, the species specific identification of pathogenic Vibrios done in this study will help the hatchery owners to improve their management and surveillance system through taking the specific actions for the specific disturbance.

5.2 Recommendations

I. To understand the *Vibrio* prevalence, samples should be drawn from more different hatcheries with information about their management strategies.

II. During the current study, the occurrence of *Vibrio species* in fish shrimp hatcheries was investigated, irrespective of season. So, further study should be conducted on seasonal occurrences in fish and shellfish.

III. The water management system of the Zomzom Hatchery should be improved and the authority should rethink about their hygienic facility as pathogenic vibrios including *V. cholerae* were found in the hatchery environment.

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Appendices

Appendix- I

Microbiological media

Media used were prepared by standard methods using appropriate compositions. Components used were high grade and were produced either by Sigma or DIFCO, USA. All media were sterilized by autoclaving for 20 minutes. The composition used for different media have been shown below

Alkaline Peptone Water (APW)

Ingredients	Amount (g/L)
Peptone	10.0
NaCl	10.0
Distilled water	1 L

pH was adjusted to 8.5 ± 0.2 after dissolving ingredients. 10 mL portions were dispensed into tubes and autoclaved for 10 minutes at 121ºC.

Luria-Bertani (LB) Broth

Ingredients	Amount (g/L)
Bacto-tryptone	10.0
Bacto-yeast extract	5.0
Sodium Chloride	10.0
Distilled water	1.0 L
pH	7.4

Marine Agar (MA) medium

Ingredients	Amount (g/L)
MA broth	42.8
Agar powder	30.0
Distilled water	1 L
pH	7.3±0.2

Nutrient Agar (NA) medium (Pelczar 1993)

Ingredients	Amount (g/L)
Beef extract	3.0
Peptone	5.0
NaCl	5.0
Agar	15.0
Distilled water	1 L
pH	7.3±0.2

Physiological Saline

Ingredients	Amount (g/L)
NaCl	9.0
Distilled water	1 litre

Thiosulphate Citrate Bile Sucrose (TCBS) agar

Ingredients	Amount (g/L)
Yeast extract	5.50
Peptone	10.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Bile	8.0
Sucrose	20.0
NaCl	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	14.0
Distilled water	1 litre
pH	8.6

Appendix- II

Laboratory reagents

Reagents, which were used in carrying out different methods together with their sources, are mentioned below:

Polymerase chain reaction (PCR) reagents

Master Mix GoTaq® (2X)

Nuclease Free Water

Forward Primer

Reverse Primer

Template DNA

*Alu*I restriction enzyme

Reaction Buffer(10x)

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04

Stored at 4ºC

PBS

Ingredients	Amount (g/1)
NaCl (sigma)	8.56
Na_2HPO_4 (sigma)	1.18
K_2HPO_4 (sigma)	0.23
KCl (sigma)	0.20
Distilled water	1.0 litre

pH was adjusted to 7.5.

10% NaCl solution

Ingredients for 100mL

Peptone	1g
NaCl	10 g
Dislilled water	100mL
pH adjusted to	7.4

Ethidium bromide solution

1.0g of ethidium bromide was dissolved in distilled in distilled water to a final volume of 100 ml. The container was wrapped in aluminum foil and stored at 4ºC.

Molecular weight marker

Tris-EDTA buffer

Gel loading dye (10X)

WX 174 RF DNA Hae fragment (Gibco, BRL)

Alsever's solution

Glucose (sigma) 2.5g

NaCl (sigma) 0.42g

Tris Na-acetate (sigma) 0.8g

Citric acid (sigma) 0.055g

Total volume was made up to 100 ml

Appendix- III

Mean bacterial density (cfu/g) with 1 SEM variability detected in the NA, MA and TCBS plates of the samples sampled from the water of the Artemia tank, shrimp PL stages from 8 to 12 and their corresponding water samples of Zomzom Hatchery, Cox's Bazar.

				LoadNA	LoadMA	LoadTCBS
Source	Artemia Tank	1		28800000.00	4800.00	3840.00
		2		24400000.00	5040.00	3360.00
		3		26000000.00	2880.00	2600.00
		4		24400000.00	2720.00	2480.00
		Total	Mean	25900000.0000	3860.0000	3070.0000
			SEM	1037625.49442	614.81705	322.23180
	Shrimp PL 8	1		2000000.00	2600.00	3200.00
		2		25600000.00	5200.00	4400.00
		Total	Mean	13800000.0000	3900.0000	3800.0000
			SEM	11800000.00000	1300.00000	600.00000
	Shrimp PL 10	1		24800000.00	2840.00	2280.00
		2		22600000.00	2280.00	2680.00
		Total	Mean	23700000.0000	2560.0000	2480.0000
			SEM	1100000.00000	280.00000	200.00000
	Shrimp PL 12	1		23200000.00	2720.00	2480.00
		2		25200000.00	3600.00	1320.00
		Total	Mean	24200000.0000	3160.0000	1900.0000
			SEM	1000000.00000	440.00000	580.00000
	Water PL Tank 8	1		2000000.00	2600.00	2120.00
		2		2800000.00	3120.00	1120.00
		Total	Mean	2400000.0000	2860.0000	1620.0000
			SEM	400000.00000	260.00000	500.00000
	Water PL Tank 10	1		2440000.00	2480.00	2880.00
		2		2880000.00	2400.00	2720.00
		Total	Mean	2660000.0000	2440.0000	2800.0000
			SEM	220000.00000	40.00000	80.00000
	Water PL Tank 12	1		2080000.00	3280.00	2200.00
		2		2080000.00	2600.00	1960.00
		Total	Mean	2080000.0000	2940.0000	2080.0000
			SEM	.00000	340.00000	120.00000

Appendix IV

Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LNNA	Between Groups	19.219	6	3.203	8.615	.003
	Within Groups	3.346	9	.372		
	Total	22.565	15			
LNMA	Between Groups	.401	6	.067	.901	.534
	Within Groups	.668	9	.074		
	Total	1.070	15			
LNTCBS	Between Groups	1.242	6	.207	3.080	.063
	Within Groups	.605	9	.067		
	Total	1.847	15			

Post Hoc Tests

Homogeneous Subsets

LNNA				
	Source	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	Water PL Tank 12	2	14.5479	
	Water PL Tank 8	2	14.6769	
	Water PL Tank 10	2	14.7904	
	Shrimp PL 8	2	15.7834	15.7834
	Shrimp PL 10	2		16.9799
	Shrimp PL 12	2		17.0010
	Artemia Tank	4		17.0674
	Sig.		.423	.385

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.154.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

LN MA			
	Source	N	Subset for alpha = 0.05
			1
Tukey HSD[a,b]	Water PL Tank 10	2	7.7996
	Shrimp PL 10	2	7.8417
	Water PL Tank 8	2	7.9544
	Water PL Tank 12	2	7.9794
	Shrimp PL 12	2	8.0485
	Shrimp PL 8	2	8.2098
	Artemia Tank	4	8.2189
	Sig.		.688
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 2.154.			
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.			

Appendix V

Mean bacterial load (cfu/g) detected in the samples of water sampled from 25 day old tilapia fry in NA and TCBS plates, in the samples of 25, 28, 33 and 40 day old tilapia fry of Reliance Tilapia Hatchery, Mymensingh.

						LoadNA	LoadTCBS
Source	Water	Age	25 day Old Fry	1		64000000.00	.00
				2		87000000.00	.00
				Total	Mean	75500000.0000	.0000
					SEM	11500000.00000	.00000
			Total	Mean		75500000.0000	.0000
				SEM		11500000.00000	.00000
	Fry	Age	25 day Old Fry	1		10000000.00	248.00
				2		26000000.00	11800.00
				3		12000000.00	580.00
				Total	Mean	16000000.0000	4209.3333
					SEM	5033222.95685	3796.54322
			28 day Old Fry	1		23200000.00	950.00
				2		10600000.00	1760.00
				3		60000000.00	12000.00
				Total	Mean	31266666.6667	4903.3333
					SEM	14819956.51516	3556.02931
			33 day Old Fry	1		80000000.00	8630.00
				2		100000000.00	12800.00
				3		80000000.00	11200.00
				Total	Mean	86666666.6667	10876.6667
					SEM	6666666.66667	1214.58269
			40 day Old Fry	1		3600000.00	5280.00
				2		8800000.00	4640.00
				3		7000000.00	11200.00
				Total	Mean	6466666.6667	7040.0000
					SEM	1524612.88347	2088.18901
			Total	Mean		35100000.0000	6757.3333
				SEM		10047568.67922	1452.02121
	Total	Mean				40871428.5714	5792.0000
		SEM				9489256.09106	1399.72762

Tests of Between-Subjects Effects

Source	Dependent Variable	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	LNNA	11.089[a]	3	3.696	11.894	.003
	LNTCBS	8.459[b]	3	2.820	1.838	.218
Intercept	LNNA	3407.134	1	3407.134	10963.440	.000
	LNTCBS	819.329	1	819.329	534.135	.000
Source	LNNA	.000	0	.	.	.
	LNTCBS	.000	0	.	.	.
Age	LNNA	11.089	3	3.696	11.894	.003
	LNTCBS	8.459	3	2.820	1.838	.218
Source * Age	LNNA	.000	0	.	.	.
	LNTCBS	.000	0	.	.	.
Error	LNNA	2.486	8	.311		
	LNTCBS	12.271	8	1.534		
Total	LNNA	3420.708	12			
	LNTCBS	840.060	12			
Corrected Total	LNNA	13.575	11			
	LNTCBS	20.731	11			

a. R Squared = .817 (Adjusted R Squared = .748)

b. R Squared = .408 (Adjusted R Squared = .186)

LNNA

	Age	N	Subset	
			1	2
Tukey HSD[a,b,c]	40 day Old Fry	3	15.6160	
	25 day Old Fry	3	16.4974	
	28 day Old Fry	3	17.0153	17.0153
	33 day Old Fry	3		18.2719
	Sig.		.060	.093

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = .311.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

LNTCBS

	Age	N	Subset
			1
Tukey HSD[a,b,c]	25 day Old Fry	3	7.0841
	28 day Old Fry	3	7.9074
	40 day Old Fry	3	8.7793
	33 day Old Fry	3	9.2813
	Sig.		.210

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 1.534.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Appendix VI

Mean bacterial build up (cfu/g) with 1 SEM of the samples drawn from the water of the live food rearing tank, pangas, magur and shing fry and their corresponding rearing pond water in NA and TCBS plates.

					NA	TCBS
Sources	Water of the live food tank	Fish	0	1	9000000.00	240.00
				2	7860000.00	184.00
				Mean	8430000.0000	212.0000
				SEM	570000.00000	28.00000
			Mean		8430000.0000	212.0000
			Std. Error of Mean		570000.00000	28.00000
	Types of fish	Fish	Pangas fry	1	120000000.00	360.00
				2	100000000.00	450.00
				Mean	110000000.0000	405.0000
				Std. Error of Mean	10000000.00000	45.00000
			magur fry	1	220000000.00	1840.00
				2	226000000.00	1720.00
				Mean	223000000.0000	1780.0000
				Std. Error of Mean	3000000.00000	60.00000
			Shing fry	1	266000000.00	1120.00
				2	288000000.00	1000.00
				Mean	277000000.0000	1060.0000
				Std. Error of Mean	11000000.00000	60.00000
			Mean		203333333.3333	1081.6667
			Std. Error of Mean		31363106.42211	252.35447
	Water of the fry pond	Fish	Pangas fry	1	30800000.00	940.00
				2	28200000.00	860.00
				Mean	29500000.0000	900.0000
				Std. Error of Mean	1300000.00000	40.00000
			magur fry	1	7100000.00	.00
				2	6420000.00	.00
				Mean	6760000.0000	.0000
				Std. Error of Mean	340000.00000	.00000
			Shing fry	1	26500000.00	400.00
				2	28000000.00	620.00
				Mean	27250000.0000	510.0000
				Std. Error of Mean	750000.00000	110.00000
			Mean		21170000.0000	470.0000
			Std. Error of Mean		4592541.05407	167.55099

2-way anova (Tests of Between-Subjects Effects)

Source	Dependent Variable	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	LNNA	18.825[a]	5	3.765	650.713	.000
	LNTCBS	5.864[b]	5	1.173	41.664	.000
Intercept	LNNA	3847.591	1	3847.591	664976.308	.000
	LNTCBS	501.960	1	501.960	17833.634	.000
Sources	LNNA	17.878	2	8.939	1544.958	.000
	LNTCBS	3.239	2	1.620	57.542	.000
Fish	LNNA	.441	2	.221	38.128	.000
	LNTCBS	1.411	2	.706	25.071	.001
Sources * Fish	LNNA	.506	1	.506	87.392	.000
	LNTCBS	1.213	1	1.213	43.095	.001
Error	LNNA	.035	6	.006		
	LNTCBS	.169	6	.028		
Total	LNNA	3866.451	12			
	LNTCBS	507.993	12			
Corrected Total	LNNA	18.860	11			
	LNTCBS	6.032	11			

a. R Squared = .998 (Adjusted R Squared = .997)

b. R Squared = .972 (Adjusted R Squared = .949)

LNNA

	Sources	N	Subset		
			1	2	3
Tukey HSD[a,b]	Water of the live food tank	2	15.9450		
	Water of the fry pond	4		17.1596	
	Types of fish	6			19.0577
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .006.

a. Uses Harmonic Mean Sample Size = 3.273.

b. Alpha = .05.

LNTCBS

	Sources	N	Subset	
			1	2
Tukey HSD[a,b]	Water of the live food tank	2	5.3478	
	Water of the fry pond	4		6.5060
	Types of fish	6		6.8153
	Sig.		1.000	.122

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .028.

a. Uses Harmonic Mean Sample Size = 3.273.

b. Alpha = .05.

Fish

LNNA

	Fish	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	0	2	15.9450			
	Pangas fry	4		17.8554		
	Shing fry	4			18.2795	
	magur fry	2				19.2226
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = .006.

a. Uses Harmonic Mean Sample Size = 2.667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

LNTCBS

	Fish	N	Subset		
			1	2	3
Tukey HSD[a,b,c]	0	2	5.3478		
	Pangas fry	4		6.3995	
	Shing fry	4		6.5875	
	magur fry	2			7.4838
	Sig.		1.000	.598	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .028.

a. Uses Harmonic Mean Sample Size = 2.667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LNNA	Between Groups	.941	2	.470	70.061	.003
	Within Groups	.020	3	.007		
	Total	.961	5			
LNTCBS	Between Groups	2.275	2	1.138	101.598	.002
	Within Groups	.034	3	.011		
	Total	2.309	5			

LNNA

	Fish	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Pangas fry	2	18.5118	
	magur fry	2		19.2226
	Shing fry	2		19.4387
	Sig.		1.000	.150

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

LNTCBS

	Fish	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Pangas fry	2	5.9977		
	Shing fry	2		6.9644	
	magur fry	2			7.4838
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LNNA	Between Groups	2.749	2	1.375	393.756	.000
	Within Groups	.010	3	.003		
	Total	2.760	5			
LNTCBS	Between Groups	.349	1	.349	6.982	.118
	Within Groups	.100	2	.050		
	Total	.449	3			

LNNA

	Water	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	magur fry	2	15.7253	
	Shing fry	2		17.1202
	Pangas fry	2		17.1989
	Sig.		1.000	.472

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Appendix-VII

16s rRNA Sequences of 8 representative strains isolated in this study

>ARH 1

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>ARH 2

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>ARH 3

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>ARH 4

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>ARH 5

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>ARH 6

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>ARH 7

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>ARH 8

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