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Isolation and Molecular Characterization of Bacteria from Diseased Farm Fish

Ahsan, Md. Kamrul

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Ph.D Thesis
Ahsan MK

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A thesis submitted to
The University of Rajshahi

For the Degree of
Doctor of Philosophy

By
Md. Kamrul Ahsan

Department of Zoology
University of Rajshahi
Rajshahi 6205
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The key to success is perseverance – and good luck.

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician, he is also a child placed before natural phenomena which impress him like a fairy tale."
- Marie Curie (1867-1934)

DECLARATION

I, Md. Kamrul Ahsan, hereby declare that the research work incorporated in the thesis entitled “ISOLATION AND MOLECULAR CHARACTERIZATION OF BACTERIA FROM DISEASED FARM FISH” is an original work was done by me and supervised by Dr. Selina Parween, Dr. Ananda Kumar Saha and Dr. Md. Mosharrof Hossain, Professors, Department of Zoology and is submitted to the University of Rajshahi for the degree of Doctor of Philosophy in Zoology. Such material has not been submitted to any other University/Institute for any degree. The literature and material that has been obtained from other sources is duly acknowledged in thesis.

(*Md. Kamrul Ahsan*)

ACKNOWLEDGEMENTS

This thesis is a culmination of a perfect working relationship with my supervisors, Professor Dr. Selina Parween, Professor Dr. Ananda Kumar Saha and Professor Dr. Md. Mosharrof Hossain, Department of Zoology, University of Rajshahi, to whom I am eternally grateful. They provided unreserved support during my research work and generously paved the way for my development as a researcher. Perhaps most importantly, I thank them for being my companion on our quest to discover what lies in the shadow of the statue.

I am also greatly indebted to the many people who in some way contributed to the progress and publication of the work contained herein. First and foremost, I thank the Chairmen of the Department of Zoology, University of Rajshahi for providing laboratory facilities. I also appreciate the help provided by Professor Dr. Aminuzzaman Md. Saleh Reza of the Department of Zoology for his valuable suggestions which make my research effort successful. I am also grateful to all the teachers of the Department of Zoology, University of Rajshahi. I am highly appreciating the help of Professor Dr. Md. Abu Reza of Genetic Engineering and Biotechnology Department, University of Rajshahi for his untiring guidance and laboratory facilities for carrying out the experiments and writing the results of molecular biology section.

Writing this thesis was not the lonely experience it could have been because of cherished friends who provided enthusiasm and empathy in just the right doses. The wonderful companionship of Dr. Moni Krishna Mohanta, Ali Mohammad Nushair, Sk. Md. Atiqur Rahman, Rokshana Ara Ruhi, and many others of the Zoology Department and Md. Akhrar-E-Ekram and Ahmed Salahuddin Kabir of Genetic Engineering and Biotechnology Department ensures that I can only think back upon the last few years with feelings of fondness and reminisce.

I am thankful to University Grants Commission (UGC) Bangladesh for bringing the research work under their fellowship programme and the Ministry of Education, Government of the Peoples' Republic of Bangladesh for providing deputation to pursue the higher education.

I am grateful to the authority of Centre for Advanced Research in Sciences (CARS), Dhaka University and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) for providing laboratory facilities to carry out genome sequencing.

The unconditional love and encouragement provided by my family served as a secure anchor during the hard and easy times; thank you.

The author

ABSTRACT

Present study was carried out for three years duration from March 2012 to February 2015 to isolate and characterize the bacteria collected from diseased farmed carp fishes and pond water. From the collected diseased carps (*Puntius sarana, Labeo rohita*, *L. bata*, *Catla catla*, *Cirrhinus mrigala*, *Hypophthalmichthys molitrix*) and water samples total nine isolates were differentiated and sub-cultured at 30-37°C in the laboratory. The bacteria were identified on the basis of their morphological, physiological and biochemical characteristics and confirmed by sequencing of 16S rRNA gene sequence analysis. Among the nine isolates six were Gram-negative rods and three were Gram-positive. Gram-negative six isolates can utilize citrate, and two isolates can ferment lactose. Four isolates were found as catalase positive, while two were weak positive; three were oxidase positive. All the nine isolates were found as fermentative type of bacteria. None of the isolates reduce sulphur, five produced indole; three were motile on motility indole urea media and showed flagellar movement on hanging-drop method. Six isolates showed positive results for methyl red test, while four of them were Voges-Proskauer positive. Sucrose was utilized by all the isolates where one of the isolate could only ferment sucrose. Among the nine isolates six showed resistance against bacitracin. All the isolates are more or less sensitive to gentamicin, neomycin, cephradine, doxycycline, tetracycline, ceftriaxone, ciprofloxacin, pefloxacin, mecillinam and nitrofurantoin. The highest colony forming units/ml was calculated as $65\text{-}71\times10^{10}$ and lowest was $11\text{-}16\times10^{8}$. Two isolates were found ampicillin resistant, and MIC for this antibiotic ranged from 320-640 µg/ml and MBC was in the range of 320-1280 µg/ml for the rest of the isolates. The MIC and MBC were ranged from 320-640 µg/ml and 320-1280 µg/ml respectively for the antibiotic tetracycline as all the isolates were sensitive to this antibiotic. Isolates showed their optimum growth in the range of temperature 28–30°C, pH 6.8–7.5 and salinity 1%. Five different primers were used and the combination of 8F–806R and 8F–1492R gave the more contrast single band in between 700–800 bp in comparison to 1kb plus DNA ladder. NCBI BLAST nucleotide sequence first matching revealed the nine isolates as *Klebsiella oxytoca* strain KB (98%), *Klebsiella oxytoca* strain SHD-1 (99%), *Klebsiella oxytoca* strain AIMST 8.Cp.16 (99%), *Pantoea* sp. strain F6-PCAi-T3P21 (84%), *Aeromonas* sp. strain OS6 (97%), *Aeromonas veronii* biovar sobria strain ER.1.24 (99%), *Brevibacillus borstelensis* strain 1CK49 (99%), *Brevibacillus borstelensis* strain 1CK49 (91%) and *Bacillus* sp. BVC99 (99%). Among the identified isolates six belongs to the family Enterobacteriaceae, two from the family Paenibacillaceae and one from the family Bacillaceae.

CONTENTS

LIST OF SYMBOLS AND ACRONYMS

%	:	percent
°C	:	degree Celsius
<	:	less than
>	:	greater than
µg	:	micro gram
µl	:	micro liter
µm	:	micrometer
ADB	:	Asian Development Bank
AHL	:	acylated homoserine lactone
AMR	:	antimicrobial agents resistance
approx.	:	approximately
ASM	:	American Society for Microbiology
AST	:	antibiotic susceptibility testing
ATCC	:	American Type Culture Collection
ATP	:	adenosine triphosphate
BBSRC	:	Biotechnology and Biological Sciences Research Council, United Kingdom
BCG	:	Bacillus Calmette Guérin
BDT	:	Bangladeshi taka
BHI	:	brain heart infusion
BKD	:	bacterial kidney disease
BLAST	:	basic local alignment search tool
bp	:	base pair
BSMY	:	basal salt media
CE	:	carp erythrodermatitis
CF	:	cystic fibrosis
CFU	:	colony forming unit
d/w	:	distilled water

DFID	:	Department for International Development
DNA	:	deoxyribonucleic acid
dNTPs	:	deoxynucleotide triphosphates
doi	:	digital object identifier
e.g.	:	exempli gratia (for example)
ECP	:	extracellular products
EDTA	:	ethylene diamino tetra acetate
EFSA	:	European Food Safety Authority
ELISA	:	enzyme linked immunosorbent assay
ERM	:	enteric redmouth
et al.	:	et alia (et alii)
etc.	:	et cetera
EU	:	European Union
EUS	:	Epizootic Ulcerative Syndrome
F	:	forward
FADH	:	flavin adenine dinucleotide
FAO	:	Food and Agriculture Organization of the United Nations
FCR	:	food conversion ratio
FEMS	:	Federation of European Microbiological Societies
g	:	gram
G+C	:	guanine + cytosine
GAPDH	:	glyceraldehyde-3-phosphate dehydrogenase
h	:	hour
HAIs	:	healthcare-associated infections
HGTs	:	horizontal genetic transfers
i.e.	:	id est (that is)
i.p	:	intraperitoneal
ICDDR,B	:	International Centre for Diarrhoeal Disease Research, Bangladesh
ICES	:	International Council for the Exploration of the Sea

ID	:	immunodeficiency
kDa	:	kiloDalton
kg	:	kilogram
l	:	liter
LAF	:	laminar air flow
LAMP	:	loop-mediated isothermal amplification
LB	:	Luria Bertani
LPS	:	lipopolysaccharides
MBC	:	minimum bactericidal concentration
MCA	:	MacConkey agar
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	milligram
MIC	:	minimum inhibitory concentration
MIU	:	motility indole urea
ml	:	milliliter
mM	:	millimole
MRVP	:	methyl red Voges-Proskauer
NA	:	nutrient agar
NACA	:	The National Advisory Committee for Aeronautics
NADH	:	nicotinamide adenine dinucleotide
NCBI	:	National Center for Biotechnology Information
NCBV	:	non culturable but viable
NIC	:	non-inhibitory concentration
OBPs	:	opportunistic bacterial pathogens
OD	:	optical density
O-F	:	oxidation-fermentation (oxidative-fermentative)
OIE	:	The Office International des Epizooties
OMP	:	outer membrane protein
PCB	:	polychlorinated biphenyls

PCR	:	polymerase chain reaction
pH	:	power (=potency) of hydrogen
PRRs	:	pathogen/pattern recognition receptors
QAC	:	Quarternary Ammonium Compound
R	:	reverse
RNA	:	ribonucleic acid
rRNA	:	ribosomal RNA
RTFS	:	rainbow trout fry syndrome
SC	:	Simmons citrate
SIM	:	sulphur indole motility
sp.	:	species
spp.	:	species (more than one)
SSTIs	:	skin and soft tissue infections
TCBS	:	thiosulphate citrate bile salts sucrose agar
TFTC	:	too few to count
TMPD	:	N, N, N′, N′-tetramethyl-p-phenylenediamine
TNTC	:	too numerous to count
TSA	:	tryptone soya agar
TSB	:	tryptone soya broth
TSI	:	triple sugar iron
U	:	unit
UDS	:	ulcerative disease syndrome
v/v	:	volume/volume
viz.	:	videlicet (that is to say, namely, to wit)
vs.	:	versus
w/v	:	weight/volume
WAHID	:	World Animal Health Information Database
WSD	:	white spot disease
YEMA	:	yeast extract mannitol agar

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প্রাণিবিদ্যা বিভাগ
রাজশাহী বিশ্ববিদ্যালয়
রাজশাহী- ৬২০৫, বাংলাদেশ

Department of Zoology
University of Rajshahi
Rajshahi- 6205, Bangladesh
Tel: +880 721 711119
Fax: +880 721 750064

CERTIFICATE

Certified that the work incorporated in the thesis "ISOLATION AND MOLECULAR CHARACTERIZATION OF BACTERIA FROM DISEASED FARM FISH" submitted by Mr. Md. Kamrul Ahsan was carried out by the candidate which was supervised by us. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(*Dr. Selina Parween*)
Principal Supervisor
Professor and Chairman
Department of Zoology
University of Rajshahi
Rajshahi 6205
Bangladesh
email: selinaparween@yahoo.com

(*Dr. Ananda Kumar Saha*)
Co-Supervisor- 1
Professor
Department of Zoology
University of Rajshahi
Rajshahi 6205
Bangladesh
email: anandroma@yahoo.com

(*Dr. Md. Mosharrof Hossain*)
Co-Supervisor- 2
Professor
Department of Zoology
University of Rajshahi
Rajshahi 6205
Bangladesh
email: mshzool@yahoo.com

CHAPTER 1: GENERAL INTRODUCTION

Introduction and literature review

Aquaculture is an important expanding enterprise in the world especially in Asian countries. Eighty four percent of the world's fish production through aquaculture comes from Asia according to FAO (1995). In spite of this, there is still a greater demand to increase the production of aquaculture products. Recently, the rapid growth of this enterprise was augmented by the development of intensification techniques in rearing aquatic animals. In practice, this means increasing the density of cultured fish populations to the tolerable maximum. This practice had resulted to increasing mass mortalities due to bacterial, viral and parasitic diseases in hatcheries and different culture systems on a scale never seen before. The annual total losses through fish diseases are largely unrecorded but are very sustainable. In Asia, currently diseases have emerged as a major constraint to the sustainable growth of aquaculture in the region (FAO 1995). It has been estimated that 15 Asian countries, having a total aquaculture output of over US$ 22.7 billion in 1990, lost US$ 1.36 billion the same year through disease (ADB/NACA 1991).

Fish plays the key role in protein supply of the people of Bangladesh. The traditional food of the nation includes rice and fish. With advancement of time, fish production from the natural freshwater bodies declined gradually and drastically due to a number of causes. However, presently pisciculture boomed up, and feeding the nation quite at acceptable price. Freshwater shrimp culture in Bangladesh opened an industrial platform and earning quite a good amount of foreign money since 1980's.

Bangladesh has a long history of rural, small-scale aquaculture, mainly based on carp culture. The culture type is either traditional, or improved traditional or polyculture. However, a number of other species have introduced in both monoculture and polyculture systems. Freshwater shrimp farming, integrated with rice cultivation and certain fish species, is also practiced in the country. Herbivorous or filter feeders such as carps have the net contribution to global fish supplies and food security (Naylor *et al*. 2000).

Global fish production has grown steadily in the last five decades, with food fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent. World per capita apparent fish consumption increased from an average of

9.9 kg in the 1960s to 19.2 kg in 2012 (Table 1.1). This impressive development has been driven by a combination of population growth, rising incomes and urbanization, and facilitated by the strong expansion of fish production and more efficient distribution channels (FAO 2014).

Aquaculture development is imbalanced and its production distribution is uneven, with Asia accounting for about 88 percent of world aquaculture production by volume. Among the leading producers, the major groups of species farmed and the farming systems vary greatly. India, Bangladesh, Egypt, Myanmar and Brazil rely very heavily on inland aquaculture of finfish while their potential for mariculture production of finfish remains largely untapped (FAO 2014).

According to the report of FAO (2014) global food fish productions from inland aquaculture and from mariculture were at the same level of 2.35 million tonnes in 1980. However, inland aquaculture growth has since outpaced mariculture growth, with average annual growth rates of 9.2 and 7.6 percent, respectively. As a result, inland aquaculture steadily increased its contribution to total farmed food fish production from 50 percent in 1980 to 63 percent in 2012. Of the 66.6 million tonnes of farmed food fish produced in 2012, two-thirds (44.2 million tonnes) were finfish species grown from inland aquaculture (38.6 million tonnes) and mariculture (5.6 million tonnes). Although finfish species grown from mariculture represent only 12.6 percent of the total farmed finfish production by volume, their value (US$23.5 billion) represents 26.9 percent of the total value of all farmed finfish species. This is because finfish grown from mariculture include a large proportion of carnivorous species, such as Atlantic salmon, trouts and groupers, that are higher in unit value than most freshwater-farmed finfish.

The rapid growth in inland aquaculture of finfish reflects the fact that it is a relatively easy-to-achieve type of aquaculture in developing countries when compared with mariculture. It now accounts for 57.9 percent of farmed food fish production globally. Freshwater fish farming makes the greatest direct contribution to the supply of affordable protein food, particularly for people still in poverty in developing countries in Asia, Africa and Latin America. This subsector is also expected, through continued promotion and sustainable development, to be the lead player in achieving long-term food and nutrition security and in meeting the increased demand for food fish by the growing population in many developing countries in the coming decades.

In 2012, 3.9 billion people, 55 percent of all humanity, lived inside the south Asian countries. The development of aquaculture has made a great contribution to the supply of food fish for consumption in most of the countries there, including several of the world's most populous countries such as China, India, Indonesia, Pakistan, Bangladesh and Japan. In 2012, the south Asian countries produced 58.3 million tonnes of food fish from aquaculture– 87.5 percent of the world's farmed food fish production. When these countries are counted together, the contribution of aquaculture to total fish production rose from 23.9 percent in 1990, to 40.2 percent in 2000, and 54.6 percent in 2012 (FAO 2014).

As at 2012, the number of species registered in FAO statistics was 567, including finfishes (354 species, with 5 hybrids), molluscs (102), crustaceans (59), amphibians and reptiles (6), aquatic invertebrates (9), and marine and freshwater algae (37). It is estimated that more than 600 aquatic species are cultured worldwide for production in a variety of farming systems and facilities of varying input intensities and technological sophistication, using freshwater, brackish water and marine water. For most farmed aquatic species, hatchery and nursery technology have been developed and established (FAO 2014).

Table 1.1 World fisheries and aquaculture production and utilization (FAO 2014).

	2007	2008	2009	2010	2011	2012
	(million tonnes)					
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	**90.8**	**90.1**	**90.1**	**89.1**	**93.7**	**91.3**
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	**49.9**	**52.9**	**55.7**	**59.0**	**62.0**	**66.6**
Total World Fisheries	**140.7**	**143.1**	**145.8**	**148.1**	**155.7**	**158.0**
UTILIZATION						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (billions)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (kg)	17.6	17.9	18.1	18.5	18.7	19.2

Food security, defined as a sustainable supply of "sufficient, affordable, nutritious and safe food, adapting to a rapidly changing world", was identified as the No. 1 research priority of the BBSRC Strategic Plan 2010–2015 (http://www.bbsrc.ac.uk/publications/ policy/ strategy/index.html).

Disease problems are occasionally observed in both pisciculture and shrimp culture systems, and these are mainly attributed to poor husbandry. However, a serious outbreak of epizootic ulcerative syndrome (EUS) occurred in 1998 in shrimp farms, and viral infections of marine shrimp also occurred in 1994 and 1996. Later on outbreak of diseases were found to occur very often in fish culture ponds. These outbreaks served to highlight the importance of aquatic animal health management in Bangladesh (Islam 2002). Intensification of culture systems, involving practices like increased stocking rates, increased feeding and fertilization programmes, which sometimes result in nutrient accumulation, algal blooms, dissolved oxygen deficiency and other water quality problems correlating with the disease problem in ponds in rural areas (Kumar 1992).

1.1 Some characteristics of modern aquaculture

The modern aquaculture is now based on some marked characteristics. Brun *et al*. (2009) mentioned these characteristics as follows:

i) Intensive and extensive production systems;

ii) Aquaculture is vital for many local/regional and national economies, both financially and employment;

iii) Traditional and novel species for culture;

iv) Continuous development of technology;

v) Increasing focus on "healthy" food production;

vi) Fish is globally the most traded food, 22% of this trade is aquaculture products which provides > 40% of the global food fish supply;

vii) Diseases have become one of the main constraints to sustainable aquaculture production and trade;

viii) Many examples of translocation of infectious agents by host movement;

ix) Increasing understanding of a need for coordinated preventive actions between stakeholders;

x) International, national and farm level biosecurity measures have become essential to control and prevent infectious diseases and their devastating economic consequences.

1.2 Bacteria and fish disease

Bangladesh is blessed with huge water resources suitable for aquaculture. Fish production is increasing, though Amin (2000) reported that production is substantially hampered due to disease. Diseases in the form of ulcer are often confused with EUS, which are more prevalent in winter.

Among the various fish diseases, bacterial infection is a major one. Bacterial disease is an illness of fish body caused by bacteria creating infection or internal disorder. It is an expression of a complex interaction between a susceptible host, a pathogen and the environment. In the presence of an infective agent in an effective number, a susceptible host suffers an infection in adverse conditions (Abowei and Briyai 2011). Bacterial diseases manifest in various ways for the impairment of the normal physiology in the host (Bassey 2011). Bacteria are single cell microscopic organisms occurring in different sizes and forms. These forms include rods, spherical, spiral, coma and filamentous shape. Bacterial organisms have cell wall, which maintains the cell integrity. Most bacterial pathogens of fish are rod shaped or flagellated, hence motile. They are psychrophylic, having a wide temperature range for growth. The pH range of bacterial growth is 5.5-10.00. Bacteria pathogens are aerobes or facultative anaerobes. Some are chromogenic, forming various pigments. A great number of them grow well on common laboratory media (Bassey 2011).

Some of the bacterial species that inhabit the aquatic environment are essential to the balance of nature with no direct consequence in causing disease in fish. Approximately 125 different bacterial species belonging to 34 different bacterial families have been associated with various fish diseases in the world (Öztürk and Altınok 2014). The list of fish pathogens keeps extending. The water used in aquaculture operations provides a natural habitat for growth and proliferation of bacteria which can be influenced by nutrient availability, pH, temperature, and other factors that affect their growth pattern, virulence, and pathogenicity. In order to grow, bacteria need an organic substrate that provide nutrients; some survive as free living organisms or exist as fish pathogens, while others are fastidious and survive indefinitely only within a host (Plumb and

Hanson 2011). Most bacteria responsible for causing disease in fish are gram negative rods but some pathogens that are gram-positive rods or cocci and a few that are acid-fast rods also cause disease in aquatic animals (Öztürk and Altınok 2014).

Bacterial diseases are responsible for heavy mortality in both wild and cultured fish. The actual role of these micro-organisms may vary from that of a primary pathogen to that of an opportunist invader of a host rendered moribund by some other disease process (Richards and Roberts 1978). The non-specific nature of diseases induced by non-fastidious and opportunistic bacterial organisms makes them unpredictable and complicates their differential diagnosis. Many of these organisms are a usual component of the bacterial flora in aquatic habitats, particularly in eutrophic systems. Stressors, often inevitable in most culture systems, predispose fish to bacterial-born diseases (Snieszko 1974).

The course of events from stress to predisposition to infection, include physiological changes described as a general alarm response syndrome. The consequences of which are to enforce barriers, normally preventing entry of bacteria to inner systems of fish and at the same time incapacitating fish defense responses and immune reactions (Mazeaud *et al*. 1977). Diagnosis of bacterial diseases of fish required observation of common clinical signs associated with the disease, isolation and identification of the causative bacterial organisms and confirmation with animal inoculation is important to fish culturist and fisheries managers (Abowei and Briyai 2011).

Disease caused by bacteria may cause heavy mortality in both wild and cultured fish, as these are found everywhere in the aquatic environment. Most bacterial pathogens are part of the normal microflora of the aquatic environment and are generally considered as secondary or opportunistic pathogens. Almost all fish bacterial pathogens are capable of independent existence outside the fish body. There are only a few obligatory pathogens, even these, however, are capable of living for a long time in the tissues of their host without causing injury. Clinical infections and disease usually occur only after the onset of some major changes in the physiology or body of the host. There has been a steady increase in the numbers of bacterial species associated with fish diseases, with new pathogens regularly recognized in the scientific literature (Austin and Austin 2007). As in all animal production systems, bacterial disease is one of the major problems facing

production, development and expansion of the aquaculture industry of the world (in Appendix table I the global outbreak of EUS is presented).

The control of disease is particularly difficult because fish are often farmed in system where production is dependent on natural environmental conditions. Changes or deterioration in the aquatic environment cause most of the bacterial diseases encountered, and environmental effects give rise to many other adverse conditions. A second major constrain on disease control is the relatively limited number of therapeutic agents available for the control of bacterial diseases agents. Even recommended therapies and preventive measures pose limitations. Their application to aquatic animals is often difficult in actual practice, and sometimes impossible to implement.

Outbreaks of major bacterial diseases in aquaculture can be significantly reduced if proper attention is paid to good husbandry practices and the maintenance of optimum environmental conditions, especially water quality. Another important consideration involves identifying the predisposing factors that may lead to a disease state. Once predisposing factors are identified, appropriate corrective measures should be initiated in the culture system.

Some bacteria produce enzymes called extracellular products (ECP) which are associated with the microorganism's virulence. ECP contain proteases, hemolysins, exohemaglutinins and cytotoxins and are highly toxic to fish. Most bacterial fish pathogens are rod-shaped, and the same species can form rough or smooth colonies depending on environmental conditions and the virulence or infective ability of the strain. Not all bacteria present in the body of a fish are pathogenic or may cause disease. Some bacteria may be harmless or even beneficial.

Bacteria are ubiquitous. This means that they can be found or are present almost everywhere in the aquatic environment. The actual role of these microorganisms may vary from being beneficial to that of being a secondary opportunistic invader, attacking only when the host is weakened or injured, or a primary pathogen that may cause the death of the species.

Fish possess a diverse array of bacterial taxa, often reflecting the composition of the microflora of the surrounding water (Austin 2006), and they are susceptible to a wide variety of bacterial pathogens especially when the fishes are physiologically unbalanced

or nutritionally deficient, or subjected to stressors, i.e., poor water quality and overstocking. Infectious diseases are the main cause of economic losses in aquaculture industry which is negatively impacted by various pathogenic organisms (Plumb 1997) such as *Edwardsiella tarda* (Gram-negative enterobacterium) which is the causative agent of edwardsiellosis in freshwater (Abdel-Lah and Shamrukh 2001). Edwardsiellosis is a septicemic disease characterized by extensive lesions in the skin, muscle and internal organs and infected commercially important fish including eels, channel catfish, mullet, Chinook salmon, flounder, carp, tilapia and striped bass (Thune *et al*. 1993). A list of bacterial fish pathogens of the world and their hosts are presented in the Appendix table II.

Most of the bacteria associated with fish diseases are naturally saprophytic organisms and widely distributed in the aquatic environment. Comparatively few species are classified as true obligate pathogens. Both groups of organisms may be present on the external body surface or in the tissues of apparently healthy fish, and their pathogenic role will only manifest itself as a consequence of stress.

Fish presented for bacteriological examination fall into two categories, diseased fish and healthy fish, although the dividing line is not always obvious. If clinically diseased fish are subjected to bacterial examination, the effort is focused either on the detection of the causative agent in external lesions such as dermal changes, changes in the gills and so on, or on the examination of the internal organs, especially kidneys, if there is suspicion of a septicaemic disease. If the presence of a specific germ (e.g. vectorship) is to be eliminated or the bacteriological examination is performed for another reason, such bacteriological examination is mainly focused on the kidneys and/or other parenchymatous tissues.

EUS is a fish disease characterized by the presence of severe, open dermal ulcers on the head, on the middle of the body, and on the dorsal regions of the fish (McGarey *et al*. 1991). EUS has been characterized as an epizootic disease of freshwater fish in the Indo-Pacific region since 1980 (FAO 1986) and was first reported in Bangladesh in 1988 (Barua *et al*. 1991). This disease is now frequently occurring in many fish farms in Bangladesh (Chowdhury 1997); the disease generally develops with ulcers that develop on the fish bodies, and the fish may die within a week of being infected. The disease has caused substantial economic loss to fish farmers and the fisheries sector. The etiological

agent(s) of EUS in Bangladesh is still unknown; however, organisms belonging to the potentially fish-pathogenic genera *Aeromonas*, *Vibrio*, *Plesiomonas*, and *Pseudomonas* were often isolated from the lesions and blood samples of infected fish. Representatives of *A. hydrophila* and *A. sobria* were recovered most frequently, followed by *Vibrio* and *Plesiomonas* spp. (McGarey *et al*. 1991).

McCarthy (1980) performed detailed experiments concerned with the ecology of *A. salmonicida* and also reviewed the work carried out by others. According to his report, contact with infected fish or contaminated water and fish farm materials, and transovarian transmission have all been cited as probable routes of infection. Also, carrier fish, which show no overt signs of disease but harbour the pathogen in their tissues, appear to be implicated in horizontal or vertical transmission. Such carrier fish are presumed to provide a reservoir which retains the pathogen in fish populations.

Although the relative numbers and types of bacteria associated with healthy fish are interesting, it is the role of these bacteria that is of importance. However, the information is generally patchy. For a start, it is relevant to inquire whether fish-associated bacteria are active metabolically or could some be inactivedormant-nonculturable (Ruby and Lee 1998). By piecing together various data, it becomes apparent that components of the bacterial microflora of fish have been associated with numerous functions.

1.2.1 Factors causing infection to fish

Infectious diseases of fishes occur when susceptible fishes are exposed to virulent pathogens under certain environmental stress conditions (Snieszko 1974). It seems that the host species is not the only significant factor in determining the species of bacterial pathogen involved, environment is another important factor also (Snieszko 1978a). The role of environment in the health and disease of fish and shellfish was described by Wedemeyer *et al*. (1976). There is a role of water, mud and detritus, contaminated implements on fish farms, animals other than fish themselves, and particularly, carrier fish, as potential sources of infection.

Under ideal environmental conditions, healthy looking fish without a clinical sign or lesion can carry pathogens that create serious risks for the spread of contagious diseases in the fish populations. Disease becomes evident only when stressful condition occurs.

Under intensive aquaculture conditions the risk of stress increases and a significant proportion of the stock may become infected. So, detection of pathogen from carrier fish is essential for the effective control of fish disease. Since most of the time, prevalence of diseases may change depending on time of the year and water temperature (Plumb 1999), it may be difficult to sample an infected fish from a population. Statistically, relevant disease surveillance and monitoring requires testing large numbers of fish (Altinok and Kurt 2003).

Water quality: Water quality in fish ponds is affected by the interactions of several chemical components like carbon dioxide, pH, alkalinity and hardness, which are interrelated and can have profound effects on pond productivity, the level of stress and fish health, oxygen availability and ammonia toxicity as well as that of certain metals. Most features of water quality are not constant, carbon dioxide and pH concentrations fluctuate daily. Alkalinity and hardness are relatively stable but can change over time, usually weeks to months, depending on the pH or mineral content of watershed and bottom soils (Wurts and Durborow 1992). Water quality and prevalence of bacterial diseases are strongly correlated. In fish ponds water quality can become a favourable environment for bacterial growth and proliferation due to the imbalance of chemical or biological factors. As a result disease outbreak can create havoc on the production of healthy fish.

Temperature and pH are limiting factors for the survival of bacteria in fish products; these facts are used during the processes of pasteurization and heat treatment, particularly of offal (Whipple and Rohovec 1994).

The aquatic environment, especially aquacultural and eutrophic waters and rivers, provides a natural habitat for growth and proliferation of bacteria because of nutrient-rich organic substrates that enhance bacterial growth. Bacterial flora of water is influenced by nutrient availability, pH, temperature, and other factors that affect their growth pattern, virulence, and pathogenicity. To grow, bacteria need an organic substrate that provide nutrients; some survive as free living organisms or serve as fish pathogens (facultative), while others are fastidious and survive indefinitely only within a host (obligate pathogens). Also, the level of salinity in water or culture media may affect growth and survival of some bacteria. Generally, an optimum pH 6 to 8 is desirable for growth of most bacteria; many die at pH above 11 or below 5. A temperature of 20–42°C is

necessary for optimum growth of most bacteria but some will grow above 50°C (thermophiles), while others grow at near 0°C (psychrophiles). Bacteria that are most prolific at temperatures of 18–45°C (mesophils) include many bacteria that are pathogenic to fish (Plumb and Hanson 2011).

1.2.2 Fish diseases in culture ponds

Motile *Aeromonas* spp. are significant causes of diseases in warm-water fish species (Starliper 2008), particularly *A. hydrophila* and *Pseudomonas fluorescens* as causative agent of bacterial septicemia (Burton *et al*. 2000).

Infectious diseases of cultured fish are the most notable constraints on the expansion of aquaculture and the realization of its full potential (Plumb 1994, Woo and Bruno 1999, Klesius *et al*. 2000, Robert and Moeller 2012). Bacterial pathogens are the most serious disease problem in fish production causing 80% of mortalities (Austin and Austin 2007). Bacterial organisms may be the primary cause of disease, or they may be secondary invaders, taking advantage of a breach in the fish's integument or compromise of its immune system. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment, whether it is freshwater or marine (Inglis *et al.* 1993, Robert and Moeller 2012). Majority of the bacterial infections are caused by Gram-negative organisms including the following pathogenic genera: *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium (Flexibacter)*, *Mycobacterium*, *Pseudomonas*, and *Vibrio*. *Streptococcus*, a Gram-positive genus, has been shown to cause disease in fishes.

Aeromonas spp. compose a large portion of the bacterial flora among freshwater aquatic organisms that are maintained in a wide range of water temperatures. *Aeromonas* organisms are Gram-negative, motile, facultative anaerobic rods that are ubiquitous in the aquatic and terrestrial environments. *A. hydrophila, A. caviae, A. sobria,* and *A. schubertii* have all been implicated in human disease and are found in association with aquatic finfish and crustaceans (Palumbo *et al*. 1989). These aeromonads frequently cause disease in cultured and pet fish. Clinical signs of *Aeromonas* infections in fish are seldom specific and include ulcerative lesions of the skin around the base of the fins and anus, raised scales, abdominal distension, and exophthalmia, all of which are signs that commonly develop with other bacterial infections. Depending on the severity of infection, anemia, hepatomegaly, and ascites may develop in affected fish.

Aeromonad infections in fish are often secondary to other stressors such as a suboptimal environment, poor water quality, parasitism, and nutritional deficiencies. The primary route for transmission to a clinician or persons handling fish is contact with mucus and tissues from infected or carrier fish. Cuts and abrasions that are already present on the hands of the handler, as well as wounds caused by handling fish directly, are possible routes of infection. Human infections with *Aeromonas* spp. often develop in immunocompromised persons for whom the bacteremia can prove life threatening (Joseph *et al*. 1979, Tsai *et al*. 2006).

Motile *Aeromonas* species occur widely in water, sludge and sewage (Kaper *et al*. 1981), the population in the environment is controlled by seasonal factors like temperature, solar radiation, phytoplankton (Monfort and Baleux 1990). In the environment, *Aeromonas* are sometimes isolated more frequently than *Escherichia coli*, however, the survival capability of *A. hydrophila* is similar to *E. coli* (Chung and Yu 1990). *Aeromonas* species are potential food-poisoning agent. *A. hydrophila* is psychrotrophic and has been associated with the spoilage of refrigerated (5°C) animal products (Majeed *et al*. 1989) and may also produce toxins at low temperature under suitable growth conditions (Krovacek *et al*. 1991, Majeed *et al*. 1989). The persistence and transmission of *Aeromonas* in a duckweed aquaculture-based hospital sewage water treatment plant in Bangladesh was studied. *Aeromonas* was found at all sites of the treatment plant, in 40% of the samples from environmental control ponds, in 8.5% of the samples from hospitalized children suffering from diarrhoea, and in 3.5% of samples from healthy humans. A significantly high number of *Aeromonas* bacteria were found in duckweed, which indicates that duckweed may serve as a reservoir for these bacteria (Rahman *et al*. 2007).

It is apparent that fish are continuously exposed to the microorganisms present in water and in sediment including the contaminants in sewage/faeces (El-Shafai *et al*. 2004). These organisms will undoubtedly influence the microflora on external surfaces, including the gills, of fish. Similarly, the digestive tract will receive water and food that is populated with microorganisms (Austin 2006).

From the published literature, it may be deduced that there are three likely scenarios for the fate of bacteria coming into contact with fish:

1. The organisms from the environment around the fish may become closely associated with and even colonise the external surfaces of the fish. There may be accumulation of the organisms at sites of damage, such as missing scales or abrasions (Austin and Austin 1987).
2. The organisms may enter the mouth with water (Olafsen 2001) or food and pass through and/or colonise the digestive tract (Austin and Austin 1987).
3. The organisms coming into contact with fish surfaces may be inhibited by the resident microflora or by natural inhibitory compounds present on or in the fish (Austin and Austin 1987).

1.2.3 Bacterial population in fish

Fish have only low bacterial populations on the skin (Horsley 1973), gill tissue has been found to harbor high bacterial populations, up to 106 bacteria g^{-1} (Trust 1975). There is anecdotal evidence that the eyes of healthy fish are devoid of bacterial colonization (Austin and Austin 1987). Muscle has been considered by some to be sterile (Apun *et al*. 1999), whereas other investigators have reported the presence of bacteria (Evelyn and McDermott 1961). Also, some workers have found bacteria in the kidney and liver of healthy fish, *Scophthalmus maximus* (turbot) (Toranzo *et al*. 1993). A consensus view is that dense bacterial populations occur in the digestive tract populations of up to ~10^8 heterotrophs g^{-1} (Trust and Sparrow 1974, Yoshimizu *et al*. 1976a,b, Campbell and Buswell 1983, Yano *et al*. 1995, Savas *et al*. 2005) and ~10^5 anaerobes g^{-1} (Trust and Sparrow 1974, Yoshimizu *et al*. 1976a,b, Kamei *et al*. 1985) have been reported with numbers appearing to be much higher than those of the surrounding water.

Some differences have been considered to reflect seasonality, with maximum and minimum counts occurring in summer and winter respectively (Yoshimizu *et al*. 1976a,b). An effect of water temperature on the size of the microflora has been reported by Diler and Diler (1998). Seasonal variation was attributed to the monsoon season, with maximal and minimal populations (Maya *et al*. 1995). Also, the population densities are likely to be influenced by the feeding regime, with fish receiving live feeds having higher populations of microflora than those feeding artificial diets (Savas *et al*. 2005). Differences in population size have been detected in specific regions of the digestive tract, even higher populations were observed in the digestive tract of juvenile compared with adult of rainbow trout and Dover sole (Lee and Lee 1995, MacDonald *et al*. 1986). In most of the cases anaerobes have been found in addition within the

intestines (Sakata *et al*. 1978). It was observed that there was an increase in bacterial populations, especially of adherent organisms, along the digestive tract (Hansen *et al*. 1992). Of course, it is likely that many organisms in the digestive tract will have been derived from the food. Some differences have been noted according to the feeding pattern of fish, detritivorous fish possessed higher bacterial populations than filter feeders (Balasubramanian *et al*. 1992).

Fish eggs may be populated by high numbers of bacteria (Yoshimizu *et al*. 1980). There is evidence that adhesion and colonisation of the egg by bacteria occurs within a few hours of fertilization (Hansen and Olafsen 1999). Undoubtedly, these organisms and those of the food and surrounding water are important for the establishment of a microflora in the digestive tract of fish larvae, incidentally, the digestive tract of newly hatched larvae contains scant bacterial populations, but is quickly colonised (Ringø and Birkbeck 1999).

(i) Surface microflora: The bacteria from the surface of freshwater fish have been reported to include *Acinetobacter johnsonii* (Gonzalez *et al*. 2000), aeromonads (notably *A. hydrophila, A. bestiarum, A. caviae, A. jandaei*, *A. schubertii*, and *A. veronii* biovar sobria (Gonzalez *et al*. 2001)), *Alcaligenes piechaudii, Enterobacter aerogenes, E. coli, Flavobacterium* (Zmyslowska *et al*. 2001), *Flexibacter* spp., *Micrococcus luteus, Moraxella* spp., *P. fluorescens*, psychrobacters (Gonzalez *et al*. 2000), and *V. fluvialis* (Diler *et al*. 2000, Allen *et al*. 1983a, Christensen 1977). To some extent, the presence of aeromonads reflected whether or not the water in which the fish occurred was polluted or clean (Gonzalez *et al*. 2001).

(ii) Gill microflora: Yellow-pigmented, Gram-negative rods, especially *Cytophaga* spp., dominate on gills (Trust 1975). Aeromonads, coryneforms, enterobacteria, Gram-positive cocci, pseudomonads, and vibrios have also been recovered from the gills of healthy juvenile rainbow trout (Nieto *et al*. 1984).

(iii) Microflora of the digestive tract: A comparatively wide range of taxa have been associated with the digestive tract of adult freshwater fish which include *Acinetobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Serratia* (Trust and Sparrow 1974), *Aeromonas* (Trust and Sparrow 1974, Yoshimizu *et al*. 1976a,b, Nieto *et al*. 1984, Ugajin 1979, Sakata *et al*. 1980), *Mycoplasma*

(Holben *et al*. 2002), *Clostridium* (Trust and Sparrow 1974) and *Fusobacterium* (Trust and Sparrow 1974, Ringø *et al*. 1995). Isolates have been identified by microplate hybridization as *A. caviae, A. hydrophila, A. jandaei, A. sobria,* and *A. veronii* (Sugita *et al*. 1994). *Alcaligenes, Eikenella* (Lee and Lee 1995), *Bacteroides* (Kamei *et al*. 1985, Sugita *et al*. 1991, 1997), *Citrobacter freundii* (Apun *et al*. 1999), *Hafnia alvei* (Ugajin 1979), *Cytophaga/Flexibacter* (Nieto *et al*. 1984), *Bacillus, Listeria, Propionibacterium, Staphylococcus* (Apun *et al*. 1999), *Moraxella* (Diler and Diler 1998), and *Pseudomonas* (Lee and Lee 1995, Apun *et al*. 1999, Nieto *et al*. 1984, Yoshimizu and Kimura 1976). In one study involving pike perch, it was concluded that *Moraxella* and *Staphylococcus* were unique to the habitat when compared with the digestive tract of other fish species (Diler and Diler 1998).

(iv) Internal organs: The liver and kidney of healthy turbot have been found to be populated by mostly *Pseudomonas* and *Vibrio*, including *V. fischeri, V. harveyi, V. pelagius*, and *V. splendidus* (Toranzo *et al*. 1993). Similarly, *Shewanella* spp. have been recovered from the internal organs (Decostere *et al*. 1996). The reasons for the presence of some of these bacteria are unclear. Moreover, it is speculative whether or not the fish are at the earliest stage of an infection cycle.

Three basic bacterial cell morphologies are spherical (coccus), rod (bacillus), and spiral shaped (spirillum). Bacteria have a particular staining characteristic (Gram's) referred to as gram positive (blue) or gram negative (red or pink). Gram-positive organisms may also be acid fast, which relates to the presence or absence of mycotic acid in the cell wall. Most bacteria responsible for fish disease are gram-negative rods but some pathogens that are gram-positive rods or cocci and a few that are acid-fast rods also cause disease in aquatic animals (Plumb and Hanson 2011).

1.3 Gross clinical signs of disease

Most bacterial disease show similar signs, especially in fishes. Bacterial infection may appear on the skin or fins of fish, in the muscles and in the internal organs. In nearly all cases, red spots, brown or black spots, or necrotic tissues can be observed. Inflammation may also occur. Proper identification of the causative agent is important to ensure successful treatment.

The appearance of every sick fish tells a story, which fits into the proverbial jigsaw pattern of disease diagnosis. Good observation uncovers many useful clues. To an extent, the external appearance of the animal may be ignored by the eager diagnostician in the rush to attack the specimen with scalpel and swabs. Formalin and bacteriological media may be the order of the day. Of course, the same is not true elsewhere in veterinary and human medicine where diagnosis is often achieved by apparently cursory glances at the wretched individual (Austin and Austin 2012).

According to Austin and Austin (2012) fish may display many behavioural and physical changes, some of which give valuable clues as to the nature of the disease. It should be emphasised, however, that many symptoms are common to a multitude of bacterial diseases. Consequently, in the following discussion symptoms have been categorised in clear groups rather than splitting them into a plethora of esoteric detail. Thus, many external signs of disease are recognised, and include:

Sluggish behaviour; twirling, spiral or erratic movement; faded pigment; darkened pigment/melanosis; eye damage– exophthalmia ('pop-eye')/corneal opacity/rupture; haemorrhaging in the eye; haemorrhaging in the mouth; erosion of the jaws/mouth; haemorrhaging in the opercula region/gills; gill damage; white nodules on the gills/skin; white spots on the head; fin rot/damage; haemorrhaging at the base of fins; haemorrhaging on the fins; tail rot/erosion; saddle-like lesions on the dorsal surface (columnaris, saddleback disease); distended abdomen developing into a distinct bloating; haemorrhaging on the surface and in the muscle; necrotising dermatitis; ulcers; external abscesses; furuncles (or boils); blood- filled blisters on the flank; protruded anus/vent; haemorrhaging around the vent; necrotic lesions on the caudal peduncle; emaciation (this should not be confused with starvation); inappetence; stunted growth; sloughing off of skin/external surface lesions; dorsal rigidity.

1.3.1 Internal abnormalities relevant to bacterial fish pathogens (after Austin and Austin 2012) apparent during post-mortem examination

The abnormalities include the following:

Skeletal deformities; gas- filled hollows in the muscle; opaqueness in the muscle; ascitic fluid in the abdominal cavity; peritonitis; petechial (pin-prick) haemorrhages on the muscle wall; haemorrhaging in the air bladder; liquid in the air bladder; white nodules (granulomas) on/in the internal organs; yellowish nodules on the internal organs;

nodules in the muscle; swollen and/or watery kidney; false membrane over the heart and/or kidney; haemorrhaging/bloody exudate in the peritoneum; swollen intestine, possibly containing yellow or bloody fluid/gastro-enteritis; intestinal necrosis and opaqueness; hyperaemic stomach; haemorrhaging in/on the internal organs; brain damage; blood in the cranium; emaciation; pale, elongated/swollen spleen; pale (possibly mottled/ discoloured) liver; yellowish liver (with hyperaemic areas); swollen liver; generalised liquefaction; the presence of tumours.

1.4 Fish pathogenic bacteria and human health

Numerous bacterial species inhabit the aquatic environment, many of which are essential to the balance of nature with no direct consequence in fish disease. However, some 60 to 70 bacterial species are capable of causing disease in aquatic animals, several of these are also infectious to humans (Plumb and Hanson 2011).

Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common depending on the season, patients' contact with fish and related environment, dietary habits and the immune system status of the exposed individual. The bacterial species which are facultatively pathogenic for both fish and man may be isolated from fish without apparent symptoms of disease. The infection source may be fish kept either for food or as a hobby (Acha and Szyfres 2001).

Novotny *et al*. (2004) gave an overview on significant bacterial causative agents of human diseases transmitted from fish used as food or by handling them. Human infections and intoxications with the following bacteria have been recorded: *Mycobacterium* spp., *Streptococcus iniae, Photobacterium damselae, V. alginolyticus, V. vulnificus, V. parahaemolyticus, V. cholerae, Erysipelothrix rhusiopathiae, E. coli, Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum, C. perfringens, Campylobacter jejuni, Delftia acidovorans, E. tarda, Legionella pneumophila*, and *Plesiomonas shigelloides*.

Mycobacteriosis is particularly significant among infections transmissible from fish to human beings, is a chronic progressive disease of fish spread all over the world, affecting sea, brackish and fresh water fish kept as in aquacultures, as in aquariums and free nature (Wolf and Smith 1999). Wayne and Kubica (1986) isolated many mycobacterial species, both rapid and slow growing, from affected fish tissues, and

S. iniae causes meningoencephalitis and death in cultured fish species (Bercovier *et al*. 1997). This species may also be an emerging human pathogen associated with injury while preparing fresh aquacultured fish.

V. vulnificus is a Gram-negative bacterium which comprises two biotypes (Tison *et al*. 1982), biotype 1 is an opportunistic human pathogen and biotype 2 was considered as an obligate eel pathogen. The bacterium is also an opportunistic pathogen for humans (Amaro *et al*. 1995) and can be transmitted by water (Amaro and Biosca 1996). The risk factors for severe *V. vulnificus* infections are chronic hepatic disease or immunodeficiency in patients (Ito *et al*. 1999, Osawa *et al*. 2002).

Aeromonas spp. has been recognized as potential foodborne pathogens for more than 20 years. Aeromonads are ubiquitous in fresh water, fish and shellfish, and also in meats and fresh vegetables (Isonhood and Drake 2002). The organism is very frequently present in many food products, including raw vegetables, and very rarely has a case been reported. Asepsis caused by *Aeromonas* is indeed dangerous (Lehane and Rawlin 2000). The same *Aeromonas* species (primarily *A. hydrophila* HG1, *A. veronii* biovar sobria HG8/10, and *A. caviae* HG4) can cause self-limiting diarrhoea, particularly in children (Kirov *et al*. 2000).

V. cholerae is often transmitted by water but fish or fish products that have been in contact with contaminated water or faeces from infected persons also frequently serve as a source of infection (Kam *et al*. 1995, Colwell 1996, Rabbani and Greenough 1999).

C. perfringens, an important cause of both food poisoning and non-food-borne diarrhoeas in humans, was found in a number of fish owing to contamination with sewage, which is the main source of this organism (Chattopadhyay 2000).

Wachsmuth and Morris (1989) confirmed the genus *Shigella* is specific host-adapted to humans and higher primates, and its presence in the environment is associated with faecal contamination. *Shigella* spp. is the cause of shigellosis (earlier name was bacillary dysentery), causing infection of the gut.

Environment is recognized as a huge reservoir for bacterial species and a source of human pathogens. Some environmental bacteria have an extraordinary range of activities that include promotion of plant growth or disease, breakdown of pollutants,

production of original biomolecules, but also multidrug resistance and human pathogenicity. The versatility of bacterial life-style involves adaptation to various niches. Adaptation to both open environment and human specific niches is a major challenge that involves intermediate organisms allowing pre-adaptation to humans.

However, reducing the environment to three general elements (water, air and soil), as opposed to the human niche, leads to the conceptual error of medical microbiology, which is to consider the environment as an abstract entity merely surrounding human beings. In fact, our environments are multiple and essentially biotic and environmental opportunistic bacterial pathogens (OBPs) naturally have a community lifestyle associated with various other organisms, which are called shelter organisms (Jackson *et al*. 2011). In both water and soil, bacteria are sheltered by invertebrates, plants and protozoa, which are recognized as hotspots for genetic exchanges and the emergence of pathogens (Jackson *et al*. 2011, Moliner *et al*. 2009). Given the current and ancient predominance of protozoa, plants and invertebrates, it is likely that bacterial interactions with shelter organisms are not only a present source of human pathogens but have also shaped bacterial evolution. Bacteria sheltered by invertebrates, protozoa and plants are equipped with factors that overcome the innate defenses of their hosts. These factors might secondarily be useful for the adaptation of OBPs to human beings and might also further the spread of novel virulence factors into existing mutualist or pathogenic bacteria (Waterfield *et al*. 2004). Hence, environmental hotspots of emergence and pre-adaptation, acting as genetic melting pots, are referred to as 'nurseries' for human OBPs (Greub and Raoult 2004, Scully and Bidochka 2006, Berg *et al*. 2005). The genus *Pantoea* among others, entertain bivalent interactions with both plants and human tissues (Rezzonico *et al*. 2009).

Regarding their pathogenicity towards human beings, bacteria are commonly classified as true (or strict) pathogens and opportunistic (or facultative) pathogens. OBPs can cause infections in patients with underlying conditions such as indwelling devices (Curtis 2008) or diseases such as cystic fibrosis (CF) (Guss *et al*. 2011). More generally, OBPs can cause disease when the host's resistance is low, whatever the reason and the duration of the host's failure. This is particularly true for healthcare-associated infections (HAIs), which cause added mortality and healthcare costs in developed countries (Klevens *et al*. 2007), and increase the burden of resource use in

the strained healthcare systems of developing countries (Lynch *et al*. 2007). In the last two decades, the impact of HAI and other opportunistic infections has notably increased; many OBPs are now being considered as emerging pathogens. Therefore, understanding the behavior and evolution of opportunistic pathogens remains a major medical challenge (Aujoulat *et al*. 2012).

The genera *Pseudomonas*, *Aeromonas* and *Ochrobactrum* provide valuable examples of opportunistic behavior associated to particular genomic structure and evolution (Aujoulat *et al*. 2012). The pragmatic approach for predicting and preventing pathogen emergence is based on epidemiological surveillance. In the future, the abundance of microbial genomic data, with an emphasis on comparative genomics, should give us a better understanding of evolution, micro-evolution and adaptation in OBPs. Hopefully this will identify traits common to all OBPs, allowing for better surveillance, prevention and treatment of the infections they cause.

Besides human and other mammals' microbiota, the environment is a huge reservoir for human pathogens. Water-, soil- and airborne pathogens, whether strict or opportunistic, represent major sources of human infections, either directly or when mediated by vectors such as food and medical devices. For instance, waterborne microbial diseases remain the leading cause of death worldwide with expanding spectrum and increasing incidence (Theron and Cloete 2002). The water-associated infectious risk is greatest for water in the close environment of human beings, such as drinking water, domestic water, recreational water and water used in healthcare. Besides the main waterborne 'true' pathogens, bacteria from aquatic habitats are frequently involved in opportunistic infections and HAIs (Sobsey and Pillai 2009).

Human pathogens recovered from fish tissue: Attention has focused on the presence of potential human pathogens in and around fish, namely *Aeromonas* spp., *C. jejuni*, *C. botulinum, C. perfringens, E. rhusiopathiae, E. tarda, L. pneumophila*, *Mycobacterium* spp., *P. damselae, P. shigelloides, S. aureus, S. iniae, V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Novotny *et al*. 2004).

Some of the bacterial pathogens, their important characters and resulted disease found widely in fish farms, are tabulated in Table 1.2.

Table 1.2 Bacterial pathogens of fin-fishes and the diseases caused by them (after Lipton *et al*. 1998).

Bacterial species	Shape	Size	Disease
Gram negative pathogens			
Vibrio anguillarum	Curved rod	2.5–3.0×1.0 µm	Vibriosis
Vibrio ordalii	Rod	2.5–3.0×1.0 µm	Vibriosis
Vibrio alginolyticus	Rod	0.6 by 1.7 µm in the logarithmic phase of growth	Vibriosis
Vibrio damsela	Curved rod (comma)	2.5 to 3.0 µm in diameter	Vibriosis
Vibrio cholerae	Curved rod (comma)	length 1.4-2.6 µm and width 0.5-0.8 µm	Vibriosis
Vibrio vulnificus	Curved rod	0.5 to 0.8 µm in diameter and 1.4 to 2.4 µm in length	Vibriosis
Aeromonas salmonicida	Rod	1.3-2.0 by 0.8-1.3 µm	Furunculosis
Aeromonas hydrophila	Straight rod with rounded ends	length 1.0-3.0 µm width 0.3-1.0 µm	Motile Aeromonad Septicaemia
Pasteurella piscicida	Rod	length 1·0–4·0 µm width 0·5–0·8 µm	Pasteurellosis
Providencia rettgeri	Rod	0.5–0.8 µm	Bacterial Haemorrhagic Septicaemia
Edwardsiella tarda	Straight short rod	length 2–3 µm and diameter 1.0 µm	Edwardsiellosis
Edwardsiella ictaluri	Rod	0.6 by 1.2-1.5 µm	Enteric Septicaemia
Yersinia ruckeri	Rod	of 0.5–0.8 µm in width and 1–3µm in length	Enteric Redmouth Disease
Acinetobacter sp.	Rod-shaped cells reverted to cocci by reduction-division when irrigated with starvation	rectangular structures were 0.1–0.2 µm in length	Acinetobacteri-osis

Table 1.2 contd.

Bacterial species	Shape	Size	Disease
Pseudomonas anguilliseptica	Rod shaped at logarithmic phase	0.7–0.9×1.9–2.3 µm at logarithmic phase	Pseudomonas Septicaemia
Pseudomonas chlororaphis	Rod	0.5–1.0×1.5–5.0 µm	Pseudomonas Septicaemia
Pseudomonas fluorescens	Rod	1×5.0–10.0 µm	Pseudomonas Septicaemia
Cytophaga psychrophila	Rod	3.0–7.0 µm long and 0.3–0.5 µm wide	Bacterial Coldwater Disease
Cytophaga spp.	Rod	2.0–4.0 µm	Fin Rot, Bacterial Gill Disease
Flexibacter columnaris	Rod	0.3-0.5 µm wide and 3-10 µm long	Columnaris
Flexibacter maritimus	Rod	0.4-0.5 µm width and 15 µm length	Flexibacteriosis
Sporocytophaga sp.	Rod (variable)	length 1 ~ 2 µm, width 0.4 ~ 0.6 µm	Salt water columnaris
Flavobacterium sp.	Long rod	0.3-0.7 µm width and 5-15 µm length	Bacterial Gill Disease
Gram positive pathogens			
Renibacterium salmoninarum	Rod (bean or pear shaped)	0.3–0.1 µm by 1.0–1.5 µm	Bacterial Kidney Disease
Eubacterium tarantellus	Rod	3.4–7.5 × 0.3–0.7 µm	Eubacterial Meningitis
Lactobacillus piscicola	Rod	1.1–1.4 × 0.5– 0.6	Pseudokidney Disease
Staphylococcus epidermidis	Coccoid (spherical)	0.5 to 1.5 µm in diameter	Staphylococcosis
Streptococcus spp.	lancet-shaped cocci	0.5 and 1.25 µm in diameter	Streptococcal Septicaemia
Clostridium botulinum	Rod	0.5-2.0 µm in width, 1.6-22.0 µm in length	Botulism
Myxococcus piscicola	conical or flask-shaped	1.0-2.0 µm long and 0.4-0.5 µm wide	White Mouth

Table 1.2 contd.

Bacterial species	Shape	Size	Disease
Acid fast pathogens			
Mycobacterium marinum	Rod	0.2 to 0.6 µm width and 1.0 to 10 µm length	Mycobacteriosis
Mycobacterium fortuitum	Rod	0.2-0.8 µm by 1.0-10.0 µm	Mycobacteriosis
Mycobacterium chelonei	Rod	about 1.0 - 2.5 µm long by 0.5 µm wide	Mycobacteriosis
Nocardia asteroides	Rod	2.0-3.0 µm in length	Nocardiosis
Nocardia kampachi	Rod	6.4–13.6 µm in length	Nocardiosis

1.5 Environment is a "Nursery" for emerging OBPs

Mutualist bacteria, also named commensal bacteria, members of the human microbiota, are good candidates for becoming OBPs because they are already adapted to grow in human beings, and to escape from and/or to be tolerated by the immune system. To a lesser extent, the situation is similar for wild or domestic mammals-associated bacteria causing anthropozoonoses (Bengis *et al*. 2004, Cutler *et al*. 2010, Pearce-Duvet 2006). Mutualist OBPs can escape from their original niche and cause a variety of infectious complications by reaching sterile or atypical anatomic sites (Mitchell 2011). They can also invade the niches, surpassing in number other members of the community, hence inducing dysbiosis (=dysbacteriosis) and local infections. Pathogenicity of mutualist strains is related to their strategies to effectively colonize their niche: strategies that can later be opportunistically used for the invasion of other anatomic niches. In the case of anthropozoonoses, large scale transmission between mammals or birds and humans can select new or emerging virulence traits that may include increased invasiveness, enhanced spread, toxin production or antimicrobial drug resistance (Cutler *et al*. 2010). The major diseases that have plagued humanity such as smallpox, influenza, tuberculosis, malaria, measles and cholera have all evolved from zoonotic infections, which shows the effectiveness in humans of virulence traits evolving over transmission of anthropozoonoses (Bengis *et al*. 2004, Cutler *et al*. 2010, Pearce-Duvet 2006). Human mutualist and anthropozoonotic agents will not be considered here except for those that are primarily environmental bacteria. In Table 1.3 the major characteristics of some selected OBPs are presented.

Table 1.3 The major characteristics of the natural behavior of selected environmental Opportunistic Bacterial Pathogen (OBP) (source: Aujoulat *et al*. 2012).

Environmental OBP	Habitat/natural host	Lifestyle	Relationships with cells	Pathogenicity for humans
Acetic Acid Bacteria	Food, Fruits, flowers Midgut, salivary glands of flying insects	Food processing Free living Symbiotic	Extracellular	HAI Diverse mild infections in ID and CF Bacteremia Chronic granulomatous disease
***Aeromonas* spp.**	Freshwater, chlorinated water Polluted soils Nematodes Mosquitoes Leeches, mollusks Fish, Amphibians, Crustacean	Free living Pathogen for fish, amphibian and mollusk	Extracellular	Diarrhoea Wound infections Bacteremia
Agrobacterium radiobacter/ Agrobacterium tumefaciens	Rhizosphere Plants	Free living Phytopathogenic	Extracellular Plant transformation	HAI Diverse mild infections in ID and CF Bacteremia
***Burkholderia cepacia* complex**	Soil Rhizosphere Plant Amoeba - *Acanthamoeba*	Free living Plant-growth promoting Phytopathogenic	Extracellular Facultative intracellular (plant, macrophage)	Infections in CF (ET-12 epidemic clone) Chronic granulomatous disease
Chromobacterium violaceum	Water and soil in tropical and subtropical ecosystems	Free living	Extracellular	Serious or fatal infections in ID and children
Legionella pneumophila	Fresh water, chlorinated water Amoeba - *Acanthamoeba* - *Naegleria* - *Echinamoeba* - *Hartmannella* Ciliata: - *Tetrahymena*	Free living Amoeba-associated	Facultative Intracellular	Serious or fatal Pneumonia (Legionellosis)
Ochrobactrum	Soil and polluted soils Rhizosphere Plants Insects Nematodes	Free living Dixeny with nematodes Plant-growth promoter Nodule formation in plant	Extracellular	HAI Diverse mild infections in ID and CF Bacteremia

Table 1.3 contd.

Environmental OBP	Habitat/natural host	Lifestyle	Relationships with cells	Pathogenicity for humans
Pseudomonas aeruginosa	Fresh and Sea water Chlorinated water Water distribution systems (hospital, domestic) Pharmaceutical water and antiseptics Wastewater Terrestrial wet ecosystems Polluted soils Rhizosphere Amoeba - *Acanthamoeba*	Free living Amoeba-associated	Extracellular Facultative intracellular in amoeba	HAI Wound infections Burn infections
Photorhabdus luminescens	*Heterorhabditis indica*	Symbiosis	Extracellular	No
Photorhabdus asymbiotica	Unknown	Non symbiotic	Facultative intracellular in macrophage	Serious soft tissue infections Bacteremia
Serratia marcescens	Plant (phloem) Insect	Free living Phytopathogen Squash bug (*Anisa tristis*)	Extracellular	HAI Ocular infections
Stenotrophomonas maltophilia	Natural water Water distribution systems (hospital, domestic) Pharmaceutical water and antiseptics Wastewater Rhizosphere Deep-sea invertebrates Food Reptiles, mammals	Free living Plant-growth promoter	Extracellular	HAI Infection in CF

Note: HAI= Healthcare-associated Infections; ID= Immunodeficiency; CF= Cystic Fibrosis

Despite the existence of shelter organisms acting as relays throughout adaptation to man, growth of bacteria of the same species or of the same genus in both environmental and human niches is a major challenge. Bacterial genome analysis and their comparative genomics reveal common traits in genome evolution, leading to pathogenicity for human beings and/or to emergence of human OBPs (Aujoulat *et al*. 2012). The authors also stated that some species of environmental bacteria have an extraordinary range of behaviors and activities, such as free life in water, breakdown

of pollutants, production of original biomolecules, beneficial effects for plant growth and health, phytopathogenic effects, association with animals and also multi-drug resistance and human pathogenicity.

1.6 *Pseudomonas aeruginosa*, the Swiss Army Knife

P. aeruginosa is a major environmental-borne human OBP, able to live in a wide range of aquatic and wet ecosystems such as rivers (Pellett *et al*. 1983), open ocean (Khan *et al*. 2007), wastewater (Schwartz *et al*. 2006), and various terrestrial environments including rhizosphere, soil from agricultural lands and hydrocarbon-polluted sites (Deziel *et al*. 1996). *P. aeruginosa* is the third cause of HAIs. Its resistance to antibiotics (Livermore 2002) and ability to thrive in wet environments have been related to its nosocomial success. Aside from HAIs, *P. aeruginosa* causes a wide range of acute and chronic human infections in predisposing situations such as chronic wounds, burn wounds and chronic obstructive pulmonary disease, particularly in cystic fibrosis (CF) patients. The colonization of this broad spectrum of habitats is enabled by metabolic versatility and a high potential for adaptation to changing environmental conditions (Frimmersdorf *et al*. 2010). The bacteria can use a variety of carbon sources, has minimal nutrient requirements, utilizes nitrogen for anaerobic respiration in addition to its preferential aerobic behavior and grows at temperatures up to 42°C. This versatility is the result of genomic and evolutionary mechanisms leading to a highly flexible repertoire of genes that ensure survival in diverse environments and expansion of niches (Aujoulat *et al*. 2012).

1.7 *Aeromonas hydrophila* and its relatives

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems such as freshwater, coastal water and sewage (Monfort and Baleux 1990). They are increasingly being reported as important pathogens for humans and for lower vertebrates, including amphibians, reptiles, and fish (Janda and Abbott 1998). These bacteria have a broad host range, and have often been isolated from humans with diarrhoea (Ishiguro and Trust 1981), as well as from fish with haemorrhagic septicaemia (Thune *et al*. 1993). The pathogenesis, pathogenic mechanism, and virulence factors responsible for selected *Aeromonas* infection in different species are not well understood. Strains isolated from the environment do not seem to differ from strains isolated from cases of infection with respect to the prevalence of virulence factors (Krovacek *et al*. 1994). However, it has

been shown that certain species are more frequently isolated from patients with diarrhoea as well as from diseased fish than from the environment (Kirov *et al*. 1994).

Aeromonads display a large spectrum of lifestyles from free-living freshwater bacteria to symbionts of a variety of blood feeder organisms, to opportunistic pathogens of fish, amphibians and human. The range of habitats is exceptionally wide, from hostile environments such as polluted or chlorinated water, to nematodes, insects, fish and mollusks, other animals and man (Janda and Abbott 2010). The genus is characterized by a large genetic and taxonomic diversity; to date 24 species. Three species *A. caviae*, *A. hydrophila* and *A. veronii*, are responsible for more than 85% of animal and human infections; while another, *A. salmonicida*, is restrictively involved in furunculosis of fish (Janda and Abbott 2010). Human infections are extremely diverse but the most frequent are skin and soft tissue infections (SSTIs), bacteremia and diarrhoea (approx. 90% of aeromonoses), (Janda and Abbott 2010). The complete genome sequence of *A. hydrophila* ATCC 7966T uncovered a broad metabolic capability and considerable potential for virulence factors, confirming its status of emerging generalist opportunistic pathogen, and earning it the nickname of 'Jack-of-all-Trades' (Seshadri *et al*. 2006). Further genome sequences of closely related species are complete (*A. veronii*, *A. salmonicida*) or in progress (*A. caviae*) and shed some light on genome evolution and adaptation related to diverse behaviours.

Aeromonads are found primarily in freshwater where virtually all species of the genus may be recovered. They have a sympatric lifestyle that favors horizontal genetic transfers (HGTs) and high genetic diversity (Georgiades and Raoult 2010). Water is the most frequent source of human infection, (i) either for SSTIs, mainly caused by *A. hydrophila* and *A. veronii*, usually at a rare frequency, except in case of natural disasters when water-borne SSTIs become explosively frequent (e.g., hurricane Katrina in New Orleans in 2005, tsunami in Thailand in 2004, earthquake in Sichuan in 2008) (Janda and Abbott 2010); (ii) or for digestive asymptomatic carriers and gastroenteritis, mostly associated with *A. caviae* and *A. veronii* (Janda and Abbott 1998), *A. caviae* being particularly associated with the environment of human beings since its density is greater in wastewater inflow than in outflow (Monfort and Baleux 1991); (iii) or for bacteremia, mainly caused by *A. caviae* and *A. veronii*, and

indirectly associated with water through the gut from where these species originates most frequently (Aujoulat *et al*. 2012).

Besides the well-known pathogens that belong to the Enterobacteriaceae family, some members such as *Providencia alcalifaciens*, *Klebsiella* spp., *Serratia* spp. and *Citrobacter* spp. have been implicated in human disease or can be the cause of opportunistic infections including bacteraemia, meningitis, urinary tract infections and wound infections, and are therefore important in clinical settings (Baylis *et al*. 2011).

1.8 Aquatic zoonoses

Lowry and Smith (2007) stated that there are some pathogens that are communicable to humans from aquatic species via consumption and can also be encountered during examination, handling, and treatment of aquatic species. The interaction of pathogens between humans and aquatic species is complex because of the various routes of transmission coupled with the fact that many of the zoonotic pathogens do not cause disease in aquatic organisms. Thus, as unaffected carriers, seemingly healthy fish have the potential to transmit pathogens to humans. It is also possible for commensal organisms that typically cause few problems for aquatic species to become a zoonotic pathogen of humans. As a further complication associated with pathogen diagnosis in fish, many clinical signs of the disease in aquatic species have little relevance to the clinical signs that develop in affected humans.

Austin and Austin (2012) stated that it should not be ignored that some fish pathogens may also on occasion cause disease in humans. Fortunately, the incidences are low, but culprits include: *A. hydrophila* (causing diarrhoea and septicaemias), *E. tarda* (diarrhoea), *M. fortuitum* (mycobacteriosis; fish tank granuloma), *M. marinum* (mycobacteriosis; fish tank granuloma), *P. damselae* (necrotising fasciitis, bacteraemia), *P. fluorescens* (wound infections), *S. iniae* (“mad fish disease”) and *V. vulnificus* (wound infections).

With regard to infections acquired through handling of aquatic organisms, there are no reported parasitic, viral, or fungal zoonoses that are derived from aquatic species exclusively through a contact route. Bacteria are the primary causative agents for zoonotic infections that develop from such contacts. Although most fish pathogens are Gram-negative bacteria, both Gram-positive and Gram-negative bacteria are among the potential

zoonotic pathogens that can be found in association with aquatic animals. *Aeromonas* spp. are more commonly associated with freshwater species, whereas *Vibrio* spp. are generally associated with marine species of aquatic organisms. The bacteria that a veterinarian may encounter are generally considered to infect humans opportunistically and diseases caused by these bacteria develop sporadically or in immunocompromised individuals. Bacteria are simply everywhere (Trust and Bartlett 1974). The world is full of bacteria; in fact, our world would not exist as we know it without them.

1.9 Aetiology of human bacterial infections

Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include mycobacteria, *S. iniae, V. vulnificus* and other *Vibrio* spp., aeromonads, *Salmonella* spp. and others (Lipp and Rose 1997, Zlotkin *et al*. 1998, Chattopadhyay 2000). People most often get infected by either direct contact to diseased fish or infected water, or by oral consumption (Acha and Szyfres 2001).

A. Infection by contact:

i) handling infected fish;

ii) after injury by cleaning aquarium with bare hands (Alinovi *et al*. 1993);

iii) any type exposure to fish pond water (Kern *et al*. 1989), especially handling tropical fish ponds (Guarda *et al*. 1992);

iv) by contact with rare tropical fish (may carry infectious bacteria) (Bhatty *et al*. 2000);

v) after injuries caused by fish spines (Said *et al*. 1998) or fish bite (Seiberras *et al*. 2000);

vi) through contact with fish living in the wild habitat (Darie *et al*. 1993);

vii) by contact with a fresh- or salt-water environment (Hayman 1991, Jernigan and Farr 2000);

viii) through processing fish in preparation of dishes and in food industry (Notermans and Hoornstra 2000).

Young children who come to the contact of fish pond very often are infected (Bleiker *et al*. 1996, Speight and Williams 1997).

B. Oral consumption: By consuming fish or related products or food contaminated with water, or other components used in the process of preparation of products and food.

Apart from factors relating to the living environment (exposure), the development of an infectious disease is markedly affected by internal factors such as the physiological status of consumer, particularly by immunosuppression and stress.

The disease in humans has three forms: (i) one of which is a localized skin infection that is generally associated with a wound, cut, or abrasion. This localized form is typically associated with the extremities, primarily the hands and fingers; (ii) the second one is a diffuse cutaneous form, which is a progression of a localized infection to surrounding tissues; (iii) the third form is systemic infection in which the heart and heart valves are targeted, resulting in endocarditis.

From the standpoint of microbiology, fish and related products are a risk foodstuff group. Particularly *C. botulinum* type E and *V. parahaemolyticus* rank among pathogenic bacteria associated with fish. Other potentially pathogenic bacteria associated with fish and shellfish include *C. perfringens*, *Staphylococcus* spp., *Salmonella* spp., *Shigella* spp., *V. cholera* and other vibrios. Outbreaks usually occur due to the ingestion of insufficiently heat-treated fish or products contaminated after/during their processing (Novotny *et al.* 2004).

1.10 Epizootiology

There has been a trend away from studying the ecology of fish pathogens, and in particular determining their role/location in the aquatic environment. Work on the NCBV state has largely ceased. This is a pity but undoubtedly reflects the lack of research funding opportunities.

In the contemporary approach toward understanding communicable diseases, epidemiology or epizootiology plays an important role in attempts to explain relationships between the hosts, pathogens, environments, and outbreaks of diseases (Wedemeyer *et al*. 1976) and the relationship can be represented in the form of an equation: $H+P+S^2=D$ (Snieszko 1978a). Where H represents the species and strain of the host, its age, and inherited susceptibility to any particular disease; P represents the pathogen causing the disease with all its variability; and S represents stress of the environment. The square of S is used because the stress caused by environment increases in geometrical progression when the conditions are approaching the limits of tolerance by the host. The D represents the disease which results if the three components

listed on the left side of the equation are in proper qualitative and quantitative proportions. In discussing bacterial diseases of aquatic animals, the role of following three factors must always be considered.

If one takes into consideration the number of species of pathogenic bacteria and the number of species of fish and shellfish that may become infected with these bacteria, the number of bacterial species known to date is relatively small (Snieszko 1978a). The specific susceptibility of aquatic animals to various bacteria can be observed only in the species that are cultured, or at least kept in captivity for awhile (Snieszko 1978a).

Pathogenicity: There has been continual interest in the determination of pathogenicity mechanisms with current focus placed on molecular approaches and the determination of relevant, virulence genes. For the future, the data from bacterial genome sequencing may be invaluable, especially as more and more taxa are examined. The reliance on the study of single cultures continues, data from which are difficult to equate with an understanding of pathogenicity at the species level. Inter-cell communication by quorum sensing signal molecules (=acylated homoserine lactones, AHL) in the regulation of some virulence factors is fascinating, with work revealing that AHLs are produced by some Gram-negative bacterial fish pathogens, notably *A. hydrophila, A. salmonicida*, *Aliivibrio salmonicida, V. splendidus, V. vulnificus* and *Y. ruckeri* (Bruhn *et al*. 2005).

In terms of pathology, there are basically two types of disease producing bacteria infectious to fish: (1) *Obligate pathogens* and (2) *nonobligate* or *facultative pathogens*. Although there are few true obligate pathogenic bacteria that cause fish diseases, *Renibacterium salmoninarum*, the etiological agent of bacterial kidney disease, and *Mycobacterium* spp. are examples rarely found in the absence of a host. However, facultative bacteria survive indefinitely in natural waters, and when environmental conditions are conducive, infectious fish diseases may result. *A. hydrophila*, a primary bacterial species involved in the motile *Aeromonas* septicemia complex, is an example of facultative bacteria (Plumb and Hanson 2011).

Fish bacterial infections can occur as a bacteremia, which implies the presence of bacterial organisms in the blood stream without clinical infection, or as a septicemia, which indicates that bacteria and toxins are actually present in the circulatory system and usually precipitate disease and clinical signs. Inflammation, hemorrhage, and

necrosis are clinical signs associated with septicemia. Pathogenic bacteria can cause disease-producing exotoxins, which is generally characteristic, but not exclusively, of gram-positive organisms. Pathogenic gram-negative bacteria produce either exotoxins or endotoxins, which consist of proteolytic enzymes that kill host cells and cause necrosis or make blood vessels more porous causing hemorrhage (Plumb and Hanson 2011). Bacterial diseases are categorized according to fish species they infect, staining reaction of the cell, or by bacterial family, genus, and species (taxonomic grouping).

The effects of environmental stress including physical and chemical pollution: There is increasing concern about the possible role of pollution in disease, particularly of wild fish stocks (Pippy and Hare 1969, Mahoney *et al*. 1973, Robohm *et al*. 1979). As this is a politically emotive issue, and particularly as the impact of climate change is in the public arena, there is likely to be an increase in monies available and, thus, a stimulation of research interest. At present, there is considerable confusion over the precise role of pollution and fish health (Bucke 1991, 1997). Nevertheless, there are good data that long-term exposure to pollutants has adversely affected the health of fish. However, mortalities among fish populations do not necessarily imply disease. Furthermore, disease may develop long after the pollutant has been effectively removed from the aquatic environment. Much of the work attempting to correlate fish disease with aquatic pollution has resulted from surveys (Dethlefsen and Watermann 1980, Dethlefsen *et al*. 1987, 2000, McVicar *et al*. 1988, Vethaak and ap Rheinallt 1992). Briefly, fish are caught with nets, and the relative incidence of disease determined. Larger numbers of diseased fish occur generally in the polluted compared to clean/unpolluted locations (Dethlefsen *et al*. 2000). However, the distinction between polluted and clean sites is imprecise. Therefore, there would be some uncertainty as to what comprises a truly polluted or clean site. Moreover, it is uncertain from surveys how long fish might have been in a polluted environment prior to capture. Thus, the effects of fish migration on the incidence of disease needs to be considered (Vethaak *et al*. 1992, Bucke *et al*. 1992, Jacquez *et al*. 1994).

Pollution has been associated with some bacterial diseases, namely fin and tail rot (Vethaak 1992, Vethaak *et al*. 1996), gill disease/hyperplasia (Kirk and Lewis 1993) and skin disease/ulceration (Vethaak 1992, Vethaak and Jol 1996). The trigger has been attributed to stressors including contaminated diets (Landsberg 1995), heavy metals e.g.

chromium (Rødsaether *et al*. 1977, Prabakaran *et al*. 2006), hydrocarbons (Khan 1987, Song *et al*. 2008), nitrogenous compounds, i.e. ammonia (Kirk and Lewis 1993) and nitrites (Hanson and Grizzle 1985), pesticides (Voigt 1994), polychlorinated biphenyls (Ekman *et al*. 2004), sewage (Austin and Stobie 1992), organic pollutants (Grawinski and Antychowicz 2001) and unspecified pollutants (Vethaak and Jol 1996). Generally, the reasons for the association between pollution and disease need to be better researched. However, proof of correlation between the occurrence of specific pollutants and disease has seldom been documented. Surveys, which have pointed to a correlation between pollution and disease, have generally not considered the nature or concentration of the pollutant(s).

There is accumulating evidence that contamination leads to a weakening of the immune state, i.e. immunosuppression (Klesius and Shoemaker 2003). One example describes the increased susceptibility of chinook salmon from a contaminated estuary to *V. anguillarum* (Arkoosh *et al*. 1998). Undoubtedly, the future will bring further examples.

1.11 Control of disease

The development of effective disease control strategies is one aspect of bacterial fish disease research that has resulted in substantial progress from new approaches to vaccination (including genetic manipulation techniques), to the use of probiotics, prebiotics, nonspecific immunostimulants and plant products. It is noteworthy that much of this work is being carried out in less developed rather than western countries. These newer approaches have been matched by a downturn of interest in the use of antimicrobial compounds insofar as there is increasing concern about the development and spread of resistance and thus a reduced efficacy against human pathogens, and tissue residues. In many countries, there is evidence of a curb on the use of antimicrobial compounds in all but human applications. However, it is accepted that elsewhere antibiotics are used extensively in aquaculture, but beware of tissue residues if the product is destined for export. In the years ahead, it is to be envisaged that there will be ever-tighter regulations on the use of antimicrobial compounds in aquaculture (Austin and Austin 2012).

Disease control strategies include better husbandry/management practices, consideration of the use of genetically disease resistant fish strains when available, the use of suitable

diets/dietary supplements, vaccines, non specific immunostimulants, probiotics, prebiotics, natural plant products, antimicrobial compounds, water disinfection, and prevention of/restriction in the movement of infected stock (Austin and Austin 2012).

It is worth remembering the age-old adage that 'prevention is better than cure', and certainly it is possible to devote more attention to preventing the occurrence of disease in fish. This is especially true for farmed fish, which tend to be at the mercy of all the extremes which their owners are capable of devising. Principally in the industrialized nations, farmed fish are subjected to questionable water quality and high stocking regimes. These are among the known prerequisites for the onset of disease cycles. Yet, the owners are among the first to seek help if anything adverse happens to the valuable stock. Fish may be reared under ideal conditions, in which case, the stock are inevitably in excellent condition without signs of disease. Careful feeding regimes are adopted, and the stocking levels are comparatively low. Much attention has been devoted to control measures. These have been categorised in Table 1.4. Although most emphasis has been placed on aquaculture, some effort has gone towards considering disease in wild fish stocks.

Table 1.4 Methods of controlling bacterial fish diseases (source: Austin and Austin 2012).

Classification of fish stocks		Disease control measures
Wild		Control of pollutants (water quality)
Farmed	1	Adequate husbandry/management practices
	2	Use of genetically resistant fish strains
	3	Suitable diets and where appropriate, use of dietary supplements
	4	Use of vaccines
	5	Use of nonspecific immunostimulants
	6	Use of probiotics/biological control
	7	Use of prebiotics/medical plant products
	8	Use of antimicrobial compounds
	9	Water treatments
	10	Preventing the movement of infected stock

The culture of finfish and shellfish are often threatened by disease causing bacteria. At times it is difficult to distinguish between infection with a pathogen and disease that may result from such infections. In addition, the stressed animals (physiological or environmental) are easily predisposed to encounter bacterial infections as well as diseases (Lipton *et al*. 1998).

The first step towards prevention and control a disease is to thoroughly understand the infectious agent/pathogen (Lipton *et al*. 1998). Retrospective work on bacterial fish pathogens carried out by different authors have indicated that 12 genera of Gram negative and 7 genera of Gram positive, in addition to 2 genera of acid fast bacteria are major bacterial fish pathogens. The list of bacterial fish pathogens and the diseases they cause are given in Table 1.2.

To ensure enhanced fish production it is needed to prevent and control all sorts of diseases and disease making factors that hamper the water quality as well as fish health. The basic knowledge about disease control depends on several subjects, *viz.* disease causing agents, how they enter into the host body, kinds and site of attack, pathogen recognition and their physiology, pathogenesis etc. Another important factor about disease control is response factors of the organism, such as, immune response and immunity, defense mechanism, ability to accumulate treatment etc.

Innate immune response is a primitive form of defense mechanism is a complex system which, in vertebrates, is composed of cellular and humoral responses. The teleost fish, has an innate immune component that shows considerable conservation with higher vertebrates particularly in mammals highlighted by the presence of orthologous pathogen/pattern recognition receptors (PRRs) and stimulated cytokines (Aoki *et al*. 2008).

Innate immunity is the first line of defense against infection and is regarded as the primeval and hence the universal form of host defense (Janeway and Medzitov 2002). Recent data suggests that animals acquire it as a product of convergent rather than divergent evolution (Ausubel 2005). Common features of the system across species include conserved receptors for microbe-recognition, antimicrobial peptides and mitogen-associated protein kinase signaling cascades. Innate immunity is generally subdivided into two parts, the cellular and humoral defense responses. Cellular

responses include the physical barrier such as mucous and epithelial tissues lining the skin, gills and stomach, which keeps infectious microorganisms from entering the body, and specialized cells (like monocytes/macrophages, granulocytes and nonspecific cytotoxic cells) capable of killing and digesting pathogens if the latter breaches the physical barriers (Aoki *et al*. 2008). These cells are recruited in the infection site primarily by inflammatory cytokines (Aoki *et al*. 2008). Humoral responses, on the other hand, employ a variety of proteins and glycoproteins capable of destroying or inhibiting growth of infectious microorganisms, which include among others anti-bacterial peptides, proteases, complement, transferrins and the antiviral myxovirus resistance-1 protein (Mx1) (Aoki *et al*. 2008).

In mammals, innate immunity is triggered by the recognition of conserved microbial products by receptors generally called pathogen recognition receptors or pattern recognition receptors (PRRs). These molecules can distinguish 'infectious nonself' from the 'noninfectious' self. PRRs, which include both extracellular and cytosolic recognition, mediate several pathways that give rise to the production of inflammatory cytokines and interferons (Lee and Kim 2007). In teleost fish, such mechanisms are believed to be conserved mainly because of the presence of PRR and cytokine orthologs (Plouffe *et al.* 2005, Stein *et al.* 2007). Furthermore, microarray analysis has revealed transcriptional modulation of various cloned fish PRRs and cytokines following bacterial agents (Kurobe *et al.* 2005, Gerwick *et al.* 2007, Peatman *et al.* 2007, 2008). On the other hand, increasing evidence also shows that there are molecules and mechanisms that are specific to teleost fish (Plouffe *et al.* 2005, Stein *et al.* 2007).

In comparisons of these with the known molecules and mechanisms in mammals, in the absence/limited functional information in teleost fish, as well as highlight recent and growing innate immune functions that are teleost fish-specific and perspectives on the basic study of fish immunology and the techniques that would help produce this knowledge, as well as the possible applications that could be generated for the improvement of aquacultural productivity (Aoki *et al*. 2008).

Knowledge of bacterial diseases would have little practical value if it did not result in improved control of these diseases. In control of fish diseases, one should avoid ideas based too much on analogies with diseases of humans and domestic animals. Fish and

shellfish are aquatic and poikilothermic, and these are important differences (Snieszko 1978a) for planning disease control techniques.

For controlling bacterial diseases in fish some biotechnological methods are presently used and new ones are in the production queue. The important biotechnological methods in controlling bacterial diseases include: development of bacterins/vaccines and also non specific immunostimulants.

Bacterins/vaccines: A vaccine could be defined as a substance that causes a specific immune response. Vaccination as a part of standard fish culture programme is relatively new although the impact of vaccination is found to be dramatic. For example, the culture of salmon in brackish water and marine environment was made possible by usage of the Vibriosis vaccine, which led to a great expansion in pen-rearing of Atlantic salmon in Norway and Chile (Leong 1993).

Considering the importance of vaccination, biotechnologists are trying to develop sub-unit vaccines, i.e., vaccines consisting of the major protective antigens of the pathogen. These types of sub-unit vaccines have evident advantages. The important advantage is that the vaccine contains only a component of the pathogen and is therefore, more chemically defined and likely to be more stable. The other advantage is that the vaccine can be produced by direct synthesis or recombinant DNA technology. Thus, these vaccines may be genetically engineered to express further protective antigens from other fish pathogens and thus yield multivalent vaccines (Lipton *et al*. 1998).

Immunostimulants: Immunostimulants are substances which elicit non-specific defense mechanisms and enhance the barrier of infections against pathogens. They are isolated from natural sources and then synthesized chemically. Cell wall preparations from bacteria, fungi, mushrooms and yeast are rich sources of immunostimulants (Lipton *et al*. 1998). They exist in various structural forms. The possible uses of immunostimulants in managing diseases on fish and shellfish have been initiated recently. Some of the common immunostimulants include: Glucans, Lectins, Lipopolysaccharides (LPS) from bacterial cell wall and wheat germ agglutinins, which are listed in Table 1.5.

Table 1.5 List of some common non-specific immunostimulants (source: Newman and Deupree 1995).

Compound	Animals tested	Results
Glucans	Prawns	Very short term
	Salmonids	Used in conjunction with alum and bacterins
	Ictalurids	Intermediate term
Peptidoglycans	Fish	Short term
Lipopolysaccharides	Larval juvenile and Adult shrimp/fish	45-120 days
Levamisole	Fish	Modest immunostimulatory protection
Quarternary Ammonium Compound (QAC)	Fish	Modest immunostimulatory protection
ISK	Fish	Modest immunostimulatory protection
Henbane	Fish	Modest immunostimulatory protection
Ascogen	Fish	Modest immunostimulatory protection

Immune enhancers are bacterial growth inhibitors which compete with pathogenic *Aeromonas* or *Vibrio* for iron (Lipton *et al*. 1998). They are also capable of preventing attachment of bacteria to the gut. If the bacteria cannot attach in the gut and multiply, they cannot secrete toxins or enter the blood system. In addition, they make bacteria to clump together which are easily expelled from the digestive system (Lipton *et al*. 1998). The enzymes are capable of destroying the bacterial cell walls and membranes selectively without damaging host cells.

The immunostimulants have several advantages:

i) being natural products, there is no environmental hazard and

ii) unlike vaccines, which give protection to a specific pathogen, immunostimulants provide a wide range of protection against several pathogens.

Most of the immunostimulants can be synthesized and the problem of residual effect on shrimps or fish is not encountered.

It is always desirable to prevent disease outbreaks and minimize the presence of pathogens rather than to treat the epizootics, once they occur. This approach is particularly important and less expensive since it can result in reduced dependence on antibiotics and other antibacterial compounds. In order to determine the cause of disease outbreak, a variety of standardized procedures are available to identify and isolate the bacterial pathogens. The methods are labour-intensive, expensive and time consuming. Considering the potential pathogenic dimensions, the following criteria provided by Lipton *et al*. (1998) are to be fulfilled before taking up further management aspects:

i) What is the sensitivity, accuracy, speed and cost compared to other procedures?

ii) Can the test be used in presumptive and/or confirmatory application?

iii) Can the test be adapted to simple field surveillance application or does it require complex laboratory facilities and equipments?

iv) Can the test be micro-modified or automated and inexpensive handling of large number of individuals and small volume samples?

v) Does the test require a destructive or a non destructive sample?

vi) Does the test require qualitative and/or quantitative results?

vii) How well do the results obtained correlate with the clinical status of the fish?

1.11.1 Vaccine and vaccination

Fish farming takes place in open waters where fish pathogens are already present. Under such conditions, avoidance is not possible, and use of chemotherapeutic drugs is expensive and transitory. Immunization is a recent and growing control measure that is proving highly promising (Anderson 1974, Fryer *et al*. 1976).

It is well known that the appearance and development of a fish disease process is the result of the interaction between pathogen, host, and environment. So, only multidisciplinary studies involving knowledge of the characteristics of the potential pathogenic microorganisms for fish, aspects of the biology of the fish hosts, as well as a better understanding of the environmental factors affecting them, will allow the application of adequate measures to prevent and control the main diseases limiting the production of freshwater and marine fishes. Regarding the infectious fish diseases caused by bacteria, although pathogenic species have been described in the majority of the existing taxonomic groups, only a relatively small number are responsible for

important economic losses in the extensive cultures worldwide (Toranzo *et al*. 2009). The authors stated that vaccination is becoming an increasingly important part of aquaculture, since it is considered a cost effective method of controlling different threatening diseases. The term vaccination strategy has been defined to include the decision as to which diseases to vaccinate against, as well as the vaccine type, vaccination method, the timing of vaccination and the use of revaccination. One important consideration for development and commercialization of vaccines includes the application methods and procedures that can be integrated into the normal production protocols of the target fish species that are relevant to the typical ecology and epidemiology of the disease (i.e. seasonal occurrence, fish size, host and geographic range of the disease). Before attempting any vaccination strategy, it is important to determine when the immune system is both morphologically and functionally mature (Toranzo *et al*. 2009).

There are many approaches, which need to be adopted in order to control bacterial disease in farmed fish (Table 1.4). These will be explained separately below.

(i) Husbandry/management

To reiterate a previous point, it is a common problem that under severe economic pressures, the aquaculturist is tempted to produce the maximum yield of fish in a finite volume of water. Some sympathy must be directed towards the fish farmers especially when prices paid for the stock are low and profit margins are inadequate. The underlying problem is that within intensive cultivation systems, the fish may be 'stressed' beyond the limit commensurate with the production of healthy specimens. The stocking density may be too high, and it has been suggested that reducing the stocking density when water temperatures are high may well prevent some diseases. Stress may be compounded by other inappropriate management practices, in which aeration and water flow are insufficient, overfeeding occurs, and hygiene declines below the threshold at which disease is more likely to ensue. It may need only one individual to act as a reservoir of infection to the rest of the stock. Unsatisfactory occurrences, which are readily controlled, include:

- the accumulation of organic matter, namely faecal material and uneaten fish food, within the fish holding facilities;
- the presence of dead fish for prolonged periods (= bad sanitation);

- the accumulation of a biofouling community, i.e. algae and slime, in the fish tanks, and the problem associated with the collapse of blooms resulting in the release of toxic materials;
- the depletion of the oxygen content of the water with a concomitant increase in nitrogen levels, especially as ammonium salts;
- the lack of proper disinfection for items entering the fish holding facilities.

Good basic hygiene (water quality) and farm husbandry practices may successfully alleviate many of the problems attributed to disease.

ii) Disinfection/water treatments

Apart from the use of antibiotics and related compounds, the application of other chemicals to water as disinfectants is effective for disease control. Such chemicals include benzalkonium chloride, chloramine B and T, chlorine, formalin and iodophors. Another approach is to alter (increase or decrease, according to the season) the temperature of water within fish holding facilities.

iii) Genetically resistant stock

This is a topic worthy of greater attention, insofar as there are numerous observations, which point to the value of genetically resistant strains or selective breeding for reducing the problems of disease. Comparative studies need to be carefully controlled so that meaningful results are obtained. It is obvious that the breeding of disease-resistant fish may be a valuable addition in the armoury of disease control in aquaculture. However, in fish farming where more than one disease is prevalent, it is not necessarily the case that a fish strain which is resistant to one disease, would similarly show resistance to others. Nevertheless, we consider that disease-resistant strains of fish have potential for areas in which diseases are enzootic. Further effort is clearly required to bring the concept to fruition.

iv) Adequate diets/dietary supplements

An area of comparatively recent interest is that of dietary influence on fish health. The precise nutritional value of commercial feeds is largely unknown. Could essential nutrient be lacking, or other compounds be present in dangerous excess? The answers are largely unknown, although it has been established that some dietary supplements may be beneficial for maintaining the health of fish. For example, Ketola (1983) highlighted a requirement for arginine and lysine by rainbow trout fry, with fin erosion

resulting from a deficiency of lysine. Medicinal plants and plant products are increasingly considered for use in aquaculture, and the evidence points to improvement in health.

v) Vaccines

The rationale for the development of fish vaccines parallels that of other aspects of veterinary and human medicine, i.e. a Utopian desire to rid fish stocks of disease coupled with a healthy regard for profit. In practical terms, the aquaculturist needs to control specific diseases which may be financially crippling in terms of high mortalities. From the opposite viewpoint, the vaccine manufacturer needs substantial (perhaps even multinational) markets in order to ensure profitability of the products. A complicating factor concerns cost of the vaccines to the user. Generally, fish farmers who produce fish for human consumption demand inexpensive, easy-to-use, reliable products; whereas the vaccine supplier needs to charge high fees, which are sufficient to recoup developing and licensing costs, pay current expenses and invest for the future. This difference in opinion between user and supplier may lead to difficulty. Moreover, with a comparatively small aquaculture industry, private vaccine manufacturers are likely to invest resources only in developing vaccines against diseases which are prevalent in many countries, rather than those restricted to small geographical areas or representing novel and emerging conditions. This attitude undermines the whole basis of prophylaxis. No easy solution is envisaged unless research costs are supported by public monies or even from the aquaculture industry itself.

vi) Composition of bacterial fish vaccines

The composition of bacterial fish vaccines may be categorised as follows:

- Chemically or heat-inactivated whole cells. These vaccines may be mono- or polyvalent. Essentially, these are the simplest, crudest and cheapest forms of fish vaccines.
- Inactivated soluble cell extracts, i.e. toxoids.
- Cell lysates.
- Attenuated live vaccines (LaFrentz *et al*. 2008), possibly genetically-engineered cells. These would be unacceptable to some regulatory authorities because of the perceived risk that the vaccine strain may revert to a pathogenic mode.

- Attenuated live, heterologous vaccines. An example is Bacillus Calmette and Guèrin (BCG), which is alive attenuated *Mycobacterium bovis* product, and protected Japanese flounder against mycobacteriosis (Kato *et al*. 2010).
- Subunit vaccines, e.g. the genes product of the *tapA* gene for the control of *A. salmonicida* infections (Nilsson *et al*. 2006).
- DNA vaccines (Pasnik and Smith 2006, Jiao *et al*. 2009, Sun *et al*. 2010).
- Purified sub-cellular components, e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), OMP and LPS. These vaccines require a detailed understanding of microbial chemistry, aspects of which are deficient for many of the bacterial fish pathogens.
- Serum, for passive immunisation (Shelby *et al*. 2002). This is largely of academic interest only, insofar as it is difficult to envisage use of the technique in the fish farm environment. A possible exception is for brood stock or pet fish.
- Mixtures of the components, detailed above.

It is difficult to identify any particular type of preparation which excels in terms of protection. Generally, the simplest approach of using inactivated whole cells has received greatest attention. This technique has been successful with a wide assortment of pathogens, including *A. hydrophila, E. ictaluri, F. columnare, P. damselae* subsp. *piscicida, V. anguillarum, V. ordalii, Aliivibrio salmonicida and Y. ruckeri.* Indeed with these pathogens (except *V. anguillarum* and *V. ordalii),* whole cell vaccines gave superior results to other more complex forms of vaccines (Austin 1984a). However, even the best vaccines do not completely prevent the occurrence of disease, necessitating the use of costly drugs to combat low levels of infection. Clearly, more research is needed, particularly in determining the precise nature of the protective antibody and of the important antigens. With this information, it may be possible to synthesise the antigens or use genetic engineering techniques to create vaccine strains suitable for inactivation in straightforward ways.

A potentially exciting and relevant development has involved the realization that some molecules may have broad spectrum use as vaccine candidates by offering protection against a range of pathogens.

vii) Antimicrobial Compounds

There is a trend away from the use of antimicrobial compounds in all non-human applications. Certainly, the use of antimicrobial compounds in fisheries remains a highly

emotive issue in which the possibility of tissue residues and the development of bacterial resistance feature prominently in any list of complaints. It is astounding that so many compounds have found use in aquaculture (Snieszko 1978b, Herwig 1979, Austin 1984b). The complete list reads like an inventory from any well-equipped pharmacy. Antibiotics, many of which are important in human medicine, appear side by side with compounds used almost exclusively in fisheries. In many instances, the introduction of a compound into fisheries use has followed closely after the initial use in human medicine. Perhaps in retrospect, it is surprising that there has not been any significant furore from the medical profession about, what could be perceived as, misuse of pharmaceutical compounds. Unfortunately, any backlash may come in the foreseeable future; therefore, it is in the interest of aquaculture that antimicrobial compounds should be carefully used.

Whatever the range of compounds available, their effectiveness is a function of the method of administration to fish (and in the way in which it is carried out). There are as many as seven basic approaches to the administration of antimicrobial compounds to fish (Table 1.6). These are the oral route via medicated food and bioencapsulation, bath, dip and flush treatments, injection and topical application. With the oral method, drugs are mixed with food, and then fed to the fish. Usually, the treatment regime leads to the administration of a unit weight of drug to a standard weight of fish per day for a pre-determined period. Examples of commonly used antimicrobial compounds have been included in Table 1.7. Fortunately, medicated food appears to be quite stable (McCracken and Fidgeon 1977). Moreover, this method is advantageous insofar as the quantities of compound fed to the fish are carefully controlled, and if sensible feeding regimes are adopted, only minimal quantities would reach the waterways. Three provisos exist, namely that:

- the fish are capable of feeding,
- the drug is palatable,
- the drug is capable of absorption intact through the gut.

Whatever the chosen method of application, drugs may be used for prevention, i.e. prophylaxis, or treatment, i.e. chemotherapy, of fish diseases. Certainly, it is comforting to note that there are treatments available for the majority of the bacterial fish

pathogens. Providing that drugs are used prudently and correctly, they will continue to offer relief from the rigours of disease for the foreseeable future.

Table 1.6 Methods for application of antimicrobial compounds to fish (source: Austin and Austin 2012).

Methods of application	Comments
Oral route (on food)	Need palatable components; minimal risk of environmental pollution
Bioencapsulation	Need palatable compounds; minimal risk of environmental pollution
Bath	Need for fairly lengthy exposure to compound, which must be soluble or capable of being adequately dispersed; problem of disposal of spent drug
Dip	Brief immersion in compound, which must be soluble or capable of being adequately dispersed; problem of disposal of dilute compound
Flush	Compound added to fish holding facility for brief exposure to fish; must be soluble or capable of being adequately dispersed; poses problem of environmental pollution
Injection	Feasible for only large and/or valuable fish; usually requires prior anaesthesia; slow; negligible risk of environmental pollution
Topical application	Feasible for treatment of ulcers on valuable/pet fish

viii) Preventing the movement and/or slaughtering of infected stock

Some diseases, e.g. BKD, ERM and furunculosis, are suspected to be spread through the movement of infected stock. Therefore, it is sensible to apply movement restrictions or even adopt a slaughter policy to diseased stock, as a means of disease control. This may prevent the spread of disease to both farmed and wild fish. Of course, the issue of movement restrictions is highly emotive among fish farmers. However, the procedure may be beneficial to the industry when viewed as a whole. To work effectively, there is a requirement for both the efficient monitoring of all stock at risk to disease, and the dissemination of the information to all interested parties. However, we believe that in any allegedly democratic society where such measures are adopted, there should be adequate compensation to the fish farmer for loss of revenue.

Table 1.7 Methods of administering some commonly used antimicrobial compounds to fish (source: Austin and Austin 2012).

Antimicrobial compound	Diseases controlled	Method(s) of administration
Acriflavine, neutral	Columnaris	5–10 mg/l in water for several hours to several days
Amoxicillin	Furunculosis, gill disease	60–80 mg/kg body weight of fish/day/10 days
Benzalkonium chloride	Fin rot, gill disease	1–2 mg/l of water for 1 h, 100 mg/l of water for 2 min
Chloramine B or T	Fin rot, gill disease, mycobacteriosis	18–20 mg/l of water at pH 7.5-8.0, treat for 2-3 days
Enrofloxacin (=Baytril)	BKD, furunculosis	10 or 20 mg/kg body weight/day/for 10 days
Erythromycin	BKD, streptococcicosis	25–100 mg/kg of fish/day for 4–21 days 20 mg of erythromycin/kg of brood stock as an injection
Florfenicol	Furunculosis, vibriosis	10 mg/kg body weight of fish/day for 10 days
Flumequine	Furunculosis, ERM, vibriosis	6 mg/kg of fish/day for 6 days
Furanace	Coldwater disease, columnaris, fin rot, gill disease, haemorrhagic septicaemia, vibriosis	(a) 2–4 mg/kg of fish/day for 3–5 days (b) 0.5–1 mg/l of water for 5–10 min, as a bath
Iodophors	Acinetobacter disease, BKD, flavobacteriosis, furunculosis, haemorrhagic septicaemia, mycobacteriosis	50–200 mg of available iodine/l of water for 10–15 min
Oxolinic acid	Columnaris, ERM, furunculosis, haemorrhagic septicaemia, vibriosis	(a) 10 mg/kg of fish/day for 10 days (b) 1 mg/l of water, as a bath for 24 h (recommended for columnaris)
Oxytetracycline	Acinetobacter disease CE, coldwater disease, columnaris, edwardsiellosis, emphysematous putrefactive disease, ERM, enteric septicaemia, fin rot, furunculosis, gill disease, haemorrhagic septicaemia, redpest, salmonid blood spot, saltwater columnaris, Streptococcicosis, ulcer disease	50–75 mg/kg of fish/day for 10 days (doses of 300 mg/kg of fish/day for indefinite periods are used to treat RTFS)
Potentiated sulphonamide	ERM, furunculosis haemorrhagic septicaemia, vibriosis	30 mg/kg of fish/day for 10 days
Sodium nifurstyrenate	Streptococcicosis	50 mg/kg of fish/day for 3–5 days

ix) Probiotics/biological control

What is the difference between a probiotic, and immunostimulant (administered orally) and an oral vaccine? The answer to this rhetorical question has not been fully considered, but there is likely to be considerable overlap between all three. Certainly, there is increasing evidence that members of the natural aquatic micro flora, including components of the fish intestinal micro flora (Fjellheim *et al*. 2007, Pérez-Sánchez *et al*. 2011), are effective at inhibiting fish pathogens, by competitive exclusion (Lalloo *et al*. 2010) which may involve the production of antibiotics or low molecular weight inhibitors. Dopazo *et al*. (1988) discovered the presence in the marine environment of antibiotic-producing bacteria, which inhibited a range of bacterial fish pathogens, including *A. hydrophila*. These inhibitors produced low molecular weight (<10 kDa) anionic, thermolabile antibiotics. Subsequently, Chowdhury and Wakabayashi (1989), Austin and Billaud (1990) and Westerdahl *et al.* (1991) reported the presence of microbial inhibitors of *F. columnare, Serratia liquefaciens* and *V. anguillarum.* Smith and Davey (1993) identified a fluorescent pseudomonad which antagonised *A. salmonicida.* Apart from the lactic acid bacteria (Pérez-Sánchez *et al*. 2011), that are mostly linked with probiotic activity in terrestrial animals, aquaculture has utilised a wide range of Gram-positive and Gram-negative bacteria, yeast, microalgae and even bacteriophages. The use of Gram-negative bacteria from genera associated with fish disease, e.g. *Aeromonas* and *Vibrio,* is of concern because of the perceived risk of the introduction of virulence genes such as by horizontal gene transfer although this has never occurred – yet! "Good" bacteria have been described for the control of numerous diseases, and there is a tendency that the probiotic works faster than an oral vaccine (Irianto and Austin 2002). The assumption that probiotics must be live preparations was dashed when it was demonstrated that formalised suspensions of cells were effective at controlling atypical *A. salmonicida* infection in goldfish (Irianto *et al*. 2003) and furunculosis in rainbow trout (Irianto and Austin 2003), when applied as feed additives. Furthermore, subcellular components, i.e. OMPs and ECPs, of probiotics were immuno-reactive with *V. harveyi* antiserum (Arijo *et al*. 2008). Along a similar theme, i.p. or i.p. injection of cell wall proteins, OMPs, LPS and whole cell proteins of two probiotics, *A. sobria* GC2 and *Bacillus subtilis* JB-1 protected rainbow trout against challenge with *Y. ruckeri* (Abbass *et al*. 2009).

Apart from competitive exclusion, probiotics work by stimulation of the innate immune response (Irianto and Austin 2003, Kim and Austin 2006) in which case they could be considered as heterologous oral vaccines, and interference with adhesion to intestinal mucosal surfaces (Chabrillón *et al*. 2005). The beneficial effect of probiotics may be further enhanced by the use of prebiotic carbohydrates, notably arabinoxylooligosaccharide, β-glucan, glucose, inulin, oligo- fructose and xylooligosaccharide that promote the growth of the "good bacteria" (Rurangwa *et al*. 2009).

In addition to the organisms mentioned above, there is a report of the benefits for disease control of using the biopesticide, *Bacillus thuringiensis* (Meshram *et al*. 1998).

1.11.2 Disease prevention

The ways of prevention and contingently of medical treatment of fish are very specific and often different from those in warm-blooded animals. They require a thorough knowledge of the environment of fish. Preventive arrangements are consisting of complicated set of treatments elaborated on the base of a good knowledge of the aetiology of disease and host (fish) biology. It concerns the elimination or restriction of infection (invasion) sources and the possibilities of its further expansion likewise the enhancement of condition of fish organism in the way to be able to withstand the infection. The prevention is of basic importance in diseases elimination. No specific therapeutics were developed for a number of diseases up to now and the result of the application of effective, experimentally verified medicaments, is often reversely affected by the operational conditions and/or the technology of rearing. The medical treatment became economically unrenumerative in this way. In addition, some treatments cannot be performed in certain periods, e.g. in growing season, during the wintering, or in some fish culture units (e.g. large ponds). That is why it is much more important to prevent from the diseases than to recover them. The effective preventive treatments are to be applied above all in specialized fish culture units with closed warm water system, in early fish fry rearing, hatcheries, carp farms, wintering ponds and storage reservoirs. Diagnosis and treatment may be difficult, especially in view of emerging antibiotic resistance in fish pathogens (Lehane and Rawlin 2000).

1.12 Evaluation of new disease

The evaluation of 'new' diseases depends on the ability to: (i) identify the cause of the 'new' disease, especially because not all diseases are caused by pathogens; (ii) develop

or validate sensitive diagnostic techniques to accurately assess the distribution of the pathogen and ascertain if other hosts are involved; (iii) trace the source (introductions, transfers, changing husbandry practices or changing environmental conditions, previously undetected 'background' infections); and (iv) determine the relative significance of host physiology, genetic and ecological factors involved in the expression of the disease (Bower and McGladdery 2003).

There are ongoing developments in the understanding of bacterial fish pathogens. New and emerging diseases are regularly recognised especially in aquaculture. Great emphasis is placed on better diagnoses, pathogenicity mechanisms, and disease control especially by immunoprophylaxis. There is an interaction between some pollutants and occurrence of fish diseases. Some fish pathogens may also cause disease of humans, and include *E. tarda*, *M. fortuitum*, *M. marinum*, *P. damselae*, *P. fluorescens*, *S. iniae* and *V. vulnificus* (Austin and Austin 2012).

With the widespread use of 16S rRNA sequencing these taxonomic changes should be more reliable than the previous reliance on phenotypic and serological methods. Over a similar timeframe, molecular methods have come to dominate the development of diagnostic procedures, vaccine development and the study of pathogenic mechanisms. However, scientists still tend to study single isolates that differ from laboratory to laboratory, and make interlaboratory comparisons difficult. The realization that not every microbial cell is capable of being cultured has come as a wakening call to science.

The recognition of new and emerging conditions: As aquaculture increases both in total production and in the range of species used, new diseases will continue to occur. In some cases, this will reflect the movement of micro-organisms from one host to another. This is likely to happen when a new fish species is introduced into an area for farming, and there is an exchange of organisms. It may be that the new species lacks resistance, and therefore infection develops. Also in large monoculture situations, it is easier for a potential pathogen to enter a weakened host, initiate a disease cycle, and spread to adjacent animals. New diseases will require research to develop effective control and diagnostic procedures (Austin and Austin 2012).

Taxonomy and diagnosis: According to Austin and Austin (2012) with the current focus on biodiversity, there has been a resurgence of interest in taxonomy although the

rush to name new taxa based around the study of single isolates is of questionable value to science. Nevertheless, the current reliance on molecular methods, and notably sequencing of the 16S rRNA gene is likely to continue; the approach has certainly improved the standard of disease diagnoses with bacterial identification losing some of its subjectivity. With the development of methods such as loop-mediated isothermal amplification (LAMP), molecular biology has become more user friendly, and may now be undertaken in routine rather than specialized laboratories. The availability of more rapid, reliable and cheaper molecular techniques will further improve diagnostic potential. In the meantime, serology offers rapid, field-based diagnostic systems.

Isolation and selective isolation of pathogens: With the advance of the molecular era with its culture-independent approaches, it is pertinent to enquire if culturing will continue to have a role in fish disease work. We argue that pure cultures logged in established culture collections provide an invaluable reference tool. However, not all organisms will grow *in vitro*; *Candidatus* provides a current challenge. Where reliance is placed on culturing as a prelude to identification then clearly suitable culture systems are needed, but there are still only a few selective isolation procedures for bacterial fish pathogens (Austin and Austin 2012).

1.13 Fish disease and economics

Fish diseases are playing one of the roles as a limiting factor in fish production and causing heavy mortalities especially in hatcheries thus affecting profit negatively. Both researchers and farmers in aquaculture area are looking for a way to get maximum amount of yield from per unit volume of water to lower the cost in aquaculture operations. One simple way to get maximum amount of yield from per unit volume of water is basically overcrowding the fish and this condition causes stress in fish which are accustomed to live freely in nature and causes unexpected losses (Tokşen 1999). Hence, it is essential that proper measurements are taken against stress causing agents such as bad water quality, bad feed nutrition, and heavy stocking rate etc. The best way to deal with diseases is to prevent both infectious and noninfectious outbreaks. Beyond that, correct diagnosis and economically acceptable treatment methods should be carried out.

1.14 Objectives of the present study

This study was therefore conducted based on the above premise and the following were its main objectives:

1. To characterize and identify the bacterial isolates based on the phenotypic properties isolated from diseased fish;
2. To know the growth characteristics of the isolates;
3. To find out the relationship of disease occurrence with hydrological and environmental parameters;
4. To know the sequence of gene coding for 16S rRNA gene of identified isolates;
5. To know the phylogenetic position of the isolated bacteria.

CHAPTER 2: MATERIALS AND METHODS

2.1 Pond selection and sampling

Now a day fish farmers as well as fish traders are very much concern about not to disclose information about their product where disease outbreak happens. They sort out diseased fishes on the harvesting spot before marketing. At the end of the year 2012, one fish producer of Kakanhat area of Rajshahi district (Figure 2.1, 2.2) personally informed that something bad happened to his farm where marketable sized carp fishes died everyday along with two big sized (about 1 kg weight) frogs. They observed that every morning at least 20 to 30 dead fishes were floating at the surface water with lesions on their skin where scale erosion occurs. After three days they harvested all the fishes and sold out. From the spot moribund fishes of different species (*Puntius sarana*, *Labeo rohita*, *L. bata*, *Catla catla*, *Cirrhinus mrigala*, *Hypophthalmichthys molitrix*) with skin lesion and scale erosion along with pale gill were brought to the Genetics and Molecular Biology Laboratory, Department of Zoology, University of Rajshahi immediately at live condition with pond water.

On July 2013 *L. rohita* with haemorrhagic skin were collected from a pond situated at Darusha of Rajshahi district (Figure 2.1, 2.2) near about 17 kilometers away from the Rajshahi city.

The third information was published in a renowned daily newspaper that fishes are dead at gill rot disease at Ullapara upazilla under Sirajganj district (Figure 2.3). On the basis of aforesaid news affected fish and pond water samples were collected from the spot with pale gill, scale erosion, reddish skin, rough whitish spot on head and ruptured caudal fin membrane of *Catla* fry.

In all the cases, bacterial swabs were taken aseptically from the skin lesions, gill, kidney, liver and slime separately of the diseased fishes and cultured on the nutrient broth (Hi-media, India) and nutrient agar (Hi-media, India) media, which was incubated for 48 hours at 37°C subjected to shaking at 120 rpm on an orbital shaker. Control plate was kept for incubation and flasks without inoculates were also prepared and incubated at 37°C with an orbital shaker. The cultures that were found turbid after a period of 0 up to 2 days were used as inocula in subsequent experiments. No bacterial growth was seen at the plate from the internal organs where same colonies were found from skin lesion

and slime. Significant growth of other isolates was found from water samples of Ullapara area. Pure cultures of the isolates were obtained from culture plate through subsequent culture. On the basis of eye observation a total of nine (9) different sized and colored colonies were selected and the isolates were maintained in slant at 4°C for further study.

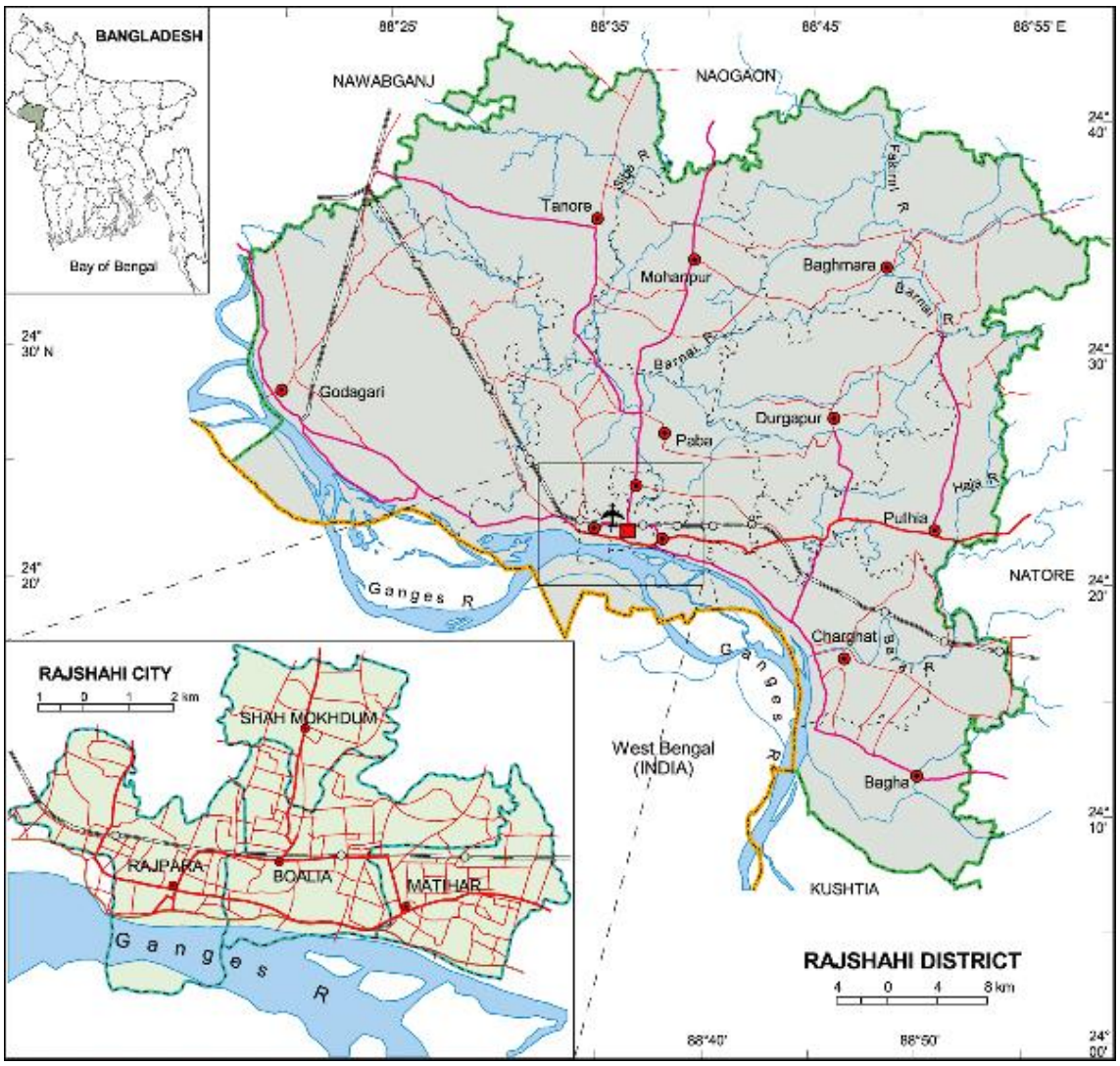


Figure 2.1 Study area of Rajshahi district (source: Google map).

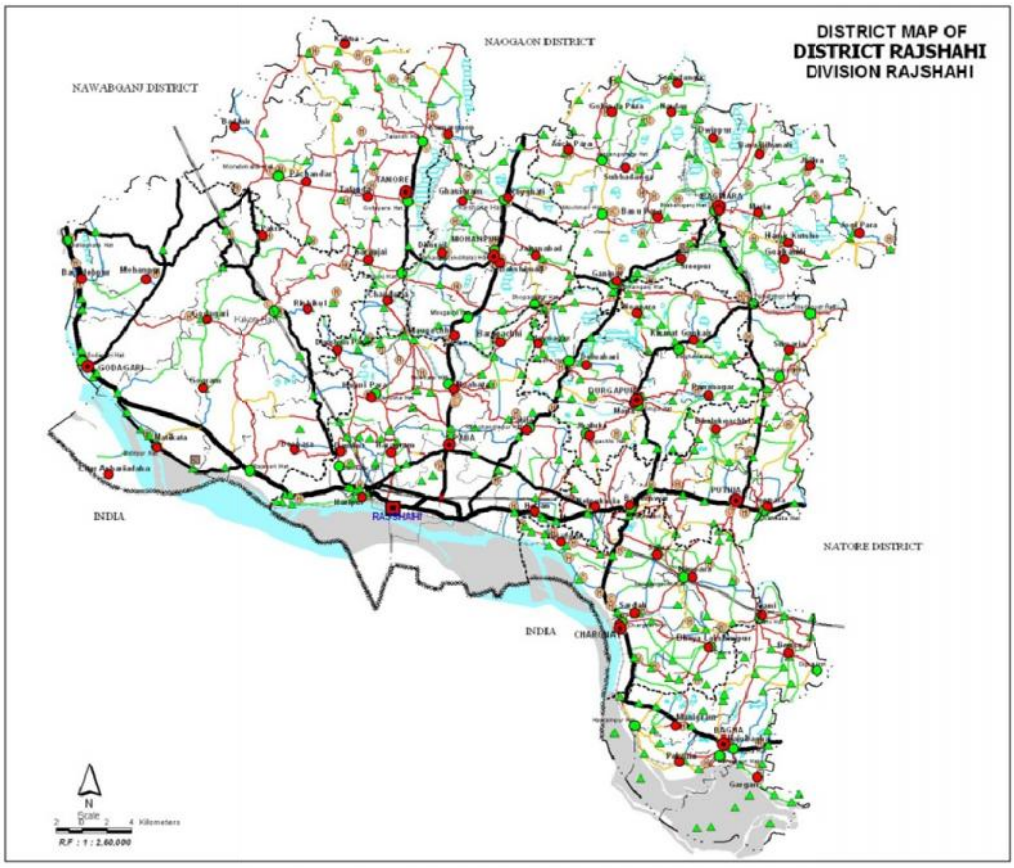


Figure 2.2 District map of Rajshahi (source: Google map).

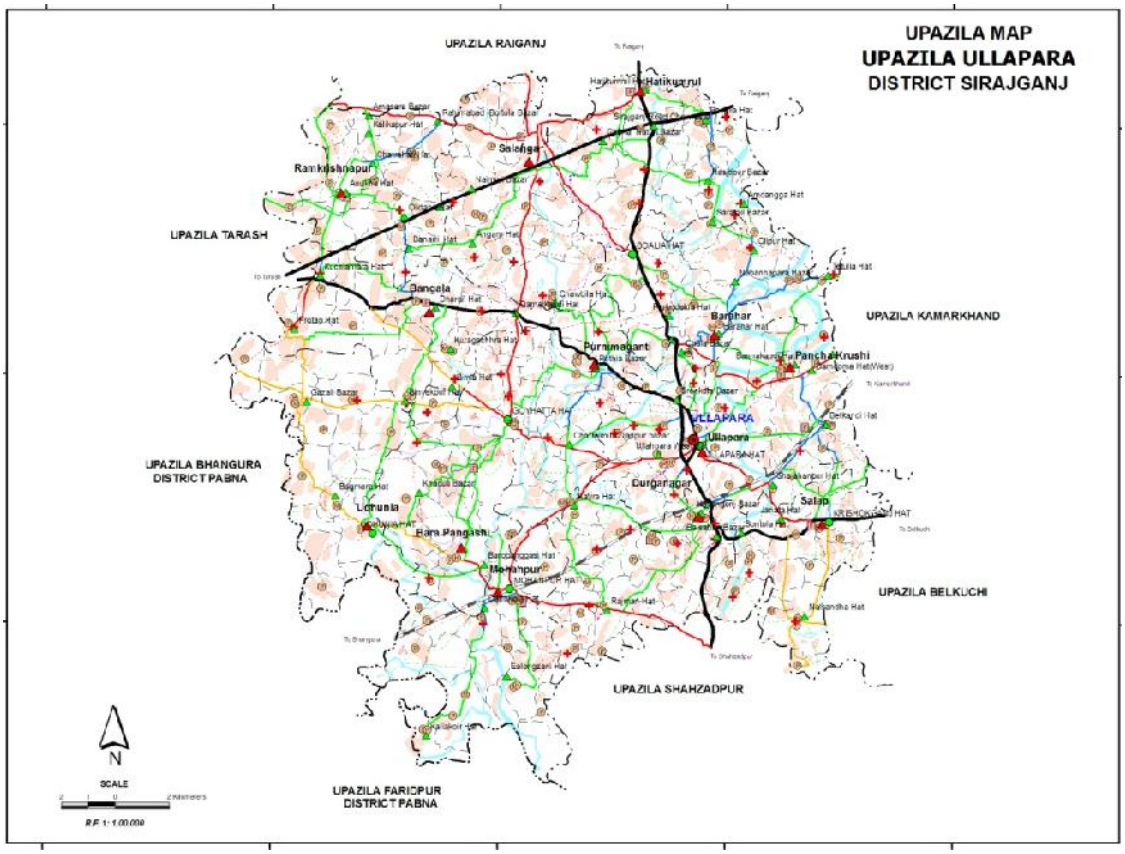


Figure 2.3 Map of Ullapara upazilla, Sirajganj (source: Google map).

The procedures of isolating bacterium were begun from environmental samples during laboratory session. Aquatic environment (culture fish pond) and fish inhabits into the water is an excellent source for microorganisms, since bacteria, algae, protozoans, yeasts, molds, and other microscopic organisms are routinely found in this environment. Initially bacteria were isolated on the primary culture plates.

The lab experiments were arranged into four exercises:

(1) a brief introduction describing the purpose of the experiments;

(2) summary tables of all unknown results;

(3) a tentative identification of the isolated bacterium using flow charts, and *Bergey's Manual of Systemic Bacteriology* (Volumes I–IV); and

(4) a conclusion discussing the basis on which the unknown was tentatively identified.

The following steps were carried out during the study.

(a) isolation of an unknown bacterium from diseased fish and pond water;

(b) identification of unknown bacterium utilizing staining techniques;

(c) determining the motility of unknown bacterium;

(d) determining the morphologic characteristics of unknown bacterium;

(e) determining the physiologic characteristics of unknown bacterium;

(f) determining the biochemical characteristics of unknown bacterium;

(g) genomic DNA isolation for 16S rRNA gene;

(h) gel electrophoresis with gDNA;

(i) polymerase chain reaction (PCR) of 16S rRNA gene;

(j) gel electrophoresis with PCR product;

(k) purification of PCR product;

(l) measurement of concentration and purity of DNA;

(m) big dye cycle sequencing;

(n) obtain sequence from automated genetic analyzer;

(o) chromatogram;

(p) NCBI BLASTn matching;

(q) clustal omega pairing;

(r) phylogenetic tree making (cladogram with distance).

Bacterial isolates were collected from ulcer type lesions on body surface such as caudal peduncle, base of fins, head lesions, internal organs like gill, liver, kidney of different fish species *viz*. *Catla*, *Labeo*, *Cirrhinus*, *Puntius*.

Nutrient agar (NA) media was used for bacteriological culture. The body surface lesions and internal organs were washed out with normal saline solution and spread on the culture plate. The petridishes were inverted and kept in incubator at 28°C and 37°C for overnight. At the following day carefully observed the plate and again different sized and coloured colonies were streaked directly with inoculating loop. The process was repeated for several times to find pure bacterial culture. For the confirmation same processes were carried out with nutrient broth.

The isolation of pure cultures on nutrient agar plates was done by streaking directly with materials previously taken, of low germ content. With the help of a nichrome wired loop, flamed to red hot and cooled, the inoculum is streaked first on the one of the 4^{th} quadrant of the plate. The inoculum taken again on the sterile loop is streaked on 2^{nd} and 3^{rd} quadrant. Finally, the inoculum taken a 4^{th} time was streaked on the last quarter. Then the petridishes were inverted and kept in incubator at 37°C for overnight. The petridishes having individual colonies in the 3^{rd} and 4^{th} streaking.

Identification of bacteria were made following the methods described in the Cowan and Steel's Manual for the identification of medical Bacteria edited by Barrow and Feltham (1993) and confirmed with the help of Bergey's Manual (Krieg and Holt 1984).

2.2 Isolation of bacteria

Before identification the bacterial pathogen should be isolated. Pure isolate is the key point of proper identification, as bacterial diseases of fish are caused by a phylogenetically diverse collection of bacterial pathogens (Sudheesh *et al*. 2012). There are a number of procedures, which are followed for the isolation of the bacteria collected from the diseased organ or organs of fish. Besides that, to study the characteristics of the pathogen a number of tests are required, to differentiate the bacterial colonies found in the diseased part. The important tests which are needed to do before identification are as follows (Table 2.1).

"Bergey's Manual" (Buchanan and Gibbons 1974) is recognized as the most authoritative text on bacterial taxonomy; however, taxonomy is becoming a separate branch of bacteriology that almost exists for its own sake.

Table 2.1 Important tests and media used in different studies.

Media	Used for	References
Tryptic Soya broth (TSB) supplemented with 3% NaCl	Growth of some suspected isolates prior to plating	Tanekhy 2013
0.5 % Nutrient agar medium supplemented with 3% NaCl	Preservation of all isolated strains as well as for the detection of bacterial motility	Tanekhy 2013
Nutrient slope agar	Preservation of bacterial isolates and for detection of pigment production by bacterial isolates	Tanekhy 2013
Trypticase Soya agar (TSA) supplemented with NaCl 3%	General purpose medium for isolation and cultivation of fastidious microorganisms	Tanekhy 2013
Blood agar medium	Isolation and cultivation of fastidious microorganisms	Whitman 2004
Thiosulphate citrate bile salts sucrose agar (TCBS) supplemented with 3% NaCl	Selective medium for the isolation of *Vibrio* species	Whitman 2004
Brain-heart infusion agar (BHI)	Isolating and cultivating a variety of (fastidious) microorganisms	Whitman 2004

2.3 Media preparation

Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties. Liquid media are used for growth of pure batch cultures while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes.

Culture media may be classified into several categories depending on their composition or use. A chemically-defined (synthetic) medium is one in which the exact chemical composition is known. A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. A defined medium is a minimal

medium if it provides only the exact nutrients (including any growth factors) needed by the organism for growth. The use of defined minimal media requires the investigator to know the exact nutritional requirements of the organisms in question. Chemically-defined media are of value in studying the minimal nutritional requirements of microorganisms, for enrichment cultures, and for a wide variety of physiological studies. Complex media usually provide the full range of growth factors that may be required by an organism so they may be more handily used to cultivate unknown bacteria or bacteria whose nutritional requirement are complex (i.e., organisms that require a lot of growth factors).

Most pathogenic bacteria of animals, which have adapted themselves to growth in animal tissues, require complex media for their growth. Blood, serum and tissue extracts are frequently added to culture media for the cultivation of pathogens. Even so, for a few fastidious pathogens artificial culture media and conditions have not been established.

An enrichment medium employs a slightly different twist. An enrichment medium contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment. However, an enrichment medium may have selective features.

A. CULTURE PLATE MEDIA

- Media were prepared in clean glassware according to manufacturer's instructions. pH was checked and adjust with 1N NaOH or HCl if necessary. Media was boiled for one minute to completely dissolve agar.

- Beaker was covered with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 30 minutes at 15 pounds pressure.

- Media was cooled to 45-50°C.

- Alternatively media was autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or water bath was used to completely melt the agar. Cool to 50°C, then proceed to next step.

- Before pouring media, hood or counter was thoroughly disinfected and sterile petri dishes were placed on the disinfected surface.

- Plates were labeled.
- Approximately 15 to 20 ml media was poured per 90×15 mm petri dish.
- Plates were inverted when the media has cooled completely to prevent excessive moisture and subsequent condensation on the plate lid.
- Plates were stored at upside down position in the refrigerator in a tightly sealed plastic bag or box.
- Manufacturer's recommendation was followed for storage period of prepared media.

B. CULTURE TUBE MEDIA

- Media was prepared in clean glassware according to manufacturer's instructions. Media was boiled if the medium contains agar, boil for one minute to completely dissolve the agar.
- pH was adjusted if the media contain any indicator. This was done when medium was at room temperature; otherwise compensation for temperature needs to be made.
- Test tubes were arranged in racks. Disposable screw cap tubes were used for all tube media.
- To dispense the medium pipette was used. Approximately 5 to 10 ml media was dispensed in 16×125mm or 20×125mm tubes and close caps loosely.
- Manufacturer's recommendation was followed for autoclave time and temperature.
- In case of slants making, tubes were kept in slant racks after autoclaving. The slant angle was adjusted to achieve the desired slant angle and butt length (i.e. short butt and long "fish-tail" slant for TSI or a standard slant over ¾ of the tube length).
- Caps were tightened when tubes can be easily handled but still warm to the touch. Cool completely to room temperature in the slanted position.
- Tubes were labeled or the tube rack with type of medium, preparers initials, and date made.
- Stored at 2-8°C, following manufacturer's recommendation for period of long-term storage.

To get pure cultures different techniques were introduced as the fish possess signs of infection at different body sites. Firstly, swabs were taken from body surface and transferred on to nutrient agar plates aseptically. Secondly, pure cultures were obtained after shaking tails and pectoral fins in 0.9% (w/v) saline solution for 5 min, and thereafter spreading 0.1 ml volumes on nutrient agar plate with incubation at 37°C for 24 to 72 hours. Thirdly, internal organs like gill, kidney and liver were washed out with normal saline solution and poured on to nutrient agar plates and incubated at 37°C for 24 to 72 hours. Pale gills of *Catla* collected from Ullapara were directly transferred in nutrient broth and kept for at least 48 hours with orbital shaker at room temperature. Where there is a lesion or somehow abnormalities seen different plates were inoculated with samples either with normal saline solution, or directly with the help of nichrome wired loop.

Isolates were periodically sub-cultured on nutrient agar and stock cultures were maintained at -20°C in nutrient broth medium containing 20% glycerol (v/v). Slants and stab were also prepared with nutrient agar as stock and kept at refrigerator at 4°C for further use. All the experimental and the reference strains were investigated at 37°C and the results were read after 24 h unless otherwise indicated.

2.4 Identification of bacterial species

Numerous species of bacteria are capable of causing diseases in fish and shellfish, many of them are well known (Bullock *et al*. 1971). Unfortunately, while trying to identify and name them a number of problems have to be confronted. Scientists worked for a long time to develop appropriate laboratory protocols for isolation and identification of bacteria.

All the tests and experiments were replicated at least three times and more (up to six or seven) whenever necessary to know their colony characteristics, morphological and physiological properties and to identify the isolated unknown bacterium based on their biochemical and molecular distinctiveness. Sometimes for many reasons findings misguide or deviate from the previous one, hence repeatedly and subsequently experiments were carried out to confirm or compare the findings. One of the possible

causes behind this may be the chance of change in the growth pattern that leads to the mutation. Present findings were compared to the previous findings from many sources such as, published literatures, manuals and books, and interpreted in the present study. Available apparatus (Appendix III) and resources were used to carry out all the experiments based on different books, and laboratory manuals (*viz*. Deshmukh 1997, Cappuccino and Sherman 1999, Noga 2000, Harley-Prescott 2002, Buller 2004, Woodland 2004, Garcia 2007, Atlas 2010).

2.4.1 Phenotypic tests

For many pathogens emphasis has been placed on conventional phenotypic tests for diagnosis, although in many laboratories there has been a rapid move towards molecular methods. Nevertheless, phenotypic methods are still used extensively. For example, Boulanger *et al*. (1977) highlighted the value of confirming the Gram-staining reaction, fermentative metabolism of glucose, and production of arginine dihydrolase, catalase and oxidase but not of lysine or ornithine decarboxylase. Caution is advocated should consideration be given to using commercially available diagnostic kits. The API 20E and API-ZYM systems, and more recently the API 20NE, API 50CH, API 50 L, Biolog-GN, Enterotubes and RapidID 32 systems (Meyer and Bullock 1973, Amandi *et al*. 1982), have made an inroad into routine diagnostic laboratories. Whereas some systems, e.g. Biolog-GN, are clearly adaptable to- and useful for environmental isolates, other have been developed specifically for a given use, usually medically important bacteria. The consequence is that the supporting identification schemes may be inappropriate. Many workers have used the API 20E system for the identification of fish pathogenic bacteria, and the technique has been considered useful be some (Santos *et al*. 1993). However, the API 20E system weighs heavily on the use of sugar fermentation reactions, which may be influenced by the presence of plasmids.

Biolog-GP2 has been regarded as effective for the identification of some Gram-positive bacterial fish pathogens when compared with the results of 16S rRNA gene sequencing (Verner-Jeffreys *et al*. 2011). Diagnoses may also be achieved also by means of diagnostic schemes based on reactions in conventional phenotypic tests. The procedure may be automated, as with the Abbott Quantum II system (Teska *et al*. 1989), and involves spectrophotometric readings at 492.6 nm, with a sample cartridge containing 20 inoculated biochemical chambers.

2.4.2 Gram staining

The technique is used for differentiation of Gram-positive and Gram-negative bacteria, usually as a first step in bacteria identification. The cell walls of Gram-positive bacteria are different from those of Gram-negative. Gram-positive walls have a thick layer of peptidoglycan associated with teichoic acids and in Gram-negative walls lipoprotein lipopolysaccharide are associated with thin peptidoglycan layer. The structural difference between cell walls results in a different ability to retain certain dyes and a different ability to resist decolorization.

There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet), followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcochol or acetone, and counterstaining with safranin. The Gram-positive cells are not discolored and remain purple. The Gram-negative cell loses its purple color. Applying safranin, Gram-negative bacteria become pink or red.

Procedural steps

1. A drop of sterilized distilled water was taken on the middle of the clean slide. A bacterial colony was taken by using inoculating loop and smear uniformly on to the slide for preparing a very thin film.
2. The smear was dried and heat fixed.
3. Smear was gently flooded with crystal violet and stand for 1 minute.
4. The slide was tilted slightly and gently rinsed with tap water or distilled water using a wash bottle.
5. The smear was gently flooded with Gram's iodine and stand for 1 minute.
6. The slide was slightly tilted and gently rinsed with tap water or distilled water using a wash bottle. The smear appeared as a purple circle on the slide.
7. Decolorized using 95% ethyl alcohol or acetone. The slide was slightly tilted and the alcohol applied drop by drop for 5 to 10 seconds until the alcohol runs almost clear.
8. The slide was immediately rinsed with water.
9. The slide was gently flooded with safranin to counter-stain and stand for 45 seconds.

10. The slide was slightly tilted and gently rinsed with tap water or distilled water using a wash bottle.
11. The slide was blot dried with bibulous paper.
12. The smear was viewed using a light-microscope under oil-immersion.

2.4.3 Potassium hydroxide string test (alternative test for Gram reaction)

A loop full of growth from a bacterial colony was emulsified on the surface of a glass slide in a suspension of 3% KOH. The suspension was stirred continuously for 60 seconds after which the loop was gently pulled from the suspension. The test was considered positive if string occurred within the first 30 seconds after mixing the bacteria in KOH solution.

Add a heavy inoculum of pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 g KOH per 100 ml distilled water) on a clean glass slide. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

Interpretation of findings:

- Gram positive bacteria did not appear to change the viscosity of the KOH solution.
- Gram negative bacteria caused the KOH solution to become stringy or mucoid in appearance and consistency.

2.4.4 Lactose fermentation test (growth on MacConkey agar)

It is a selective and differential medium for the detection of coliform organisms and enteric pathogens according to PH EUR (Agar Medium H). MacConkey agar (MCA) is a widely used culture medium that is both selective and differential. A selective medium selects for the growth of some organisms, while inhibiting the growth of others. In the case of MAC agar, the presence of bile salts and crystal violet inhibits the growth of most Gram positive bacteria. A differential medium does not inhibit the growth of bacteria, but differentiates them based on some visible growth characteristic such as colony color. MAC agar contains lactose, a fermentable carbohydrate, and the pH indicator neutral red.

When lactose is fermented, acid products lower the pH below 6.8 with the resulting colonial growth turning pinkish-red. If an organism is unable to ferment lactose, the colonies will be colorless or yellow. The medium thus differentiates between lactose-fermenting bacteria and lactose non-fermenters, which include potential pathogens.

MAC agar is a commonly used primary plating medium in many clinical microbiology laboratories. Since this medium is so common, and because it can provide timely clues as to the identification of some Gram-negative bacilli, it behooves microbiologists to be efficient in interpreting colonial growth. It will be especially helpful in the Unknown Identification lab in the differentiation of Gram positive and Gram negative unknowns.

Formulation:

Pancreatic digest of gelatin	17 g/l
Peptones	3 g/l
Bile salts	1.5 g/l
Lactose monohydrate	10 g/l
Sodium chloride	5 g/l
Neutral red	0.03 g/l
Crystal violet	0.001 g/l
Agar	13.5 g/l
pH	7.4

Interpretation of findings:

- Growth occurred and all colonies are noticeably pinkish red. Acid has been produced- Lactose fermenter
- Growth occurred and colonies are colorless. No acid has been produced- Lactose non-fermenter
- No growth occurred. Bile salts and crystal violet in the medium inhibited growth of the organism.

2.4.5 Citrate utilization test (growth on Simmons citrate agar)

It is a differential medium for the differentiation of gram negative enteric bacteria on the basis of citrate utilization. The pH indicator in Simmons Citrate (SC) agar, bromothymol blue, will turn from green from acidic conditions to a royal blue when the medium becomes alkaline.

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of *Enterobacteriaceae*. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO_2. If CO_2 is produced, it reacts with components of the medium to produce an alkaline compound (e.g. Na_2CO_3). The alkaline pH turns the pH indicator (bromothymol blue) from green to blue.

Formulation:

Sodium chloride	5.0 g/l
Sodium citrate	2.0 g/l
Magnesium sulphate	0.2 g/l
Bromothymol blue (BTB)	0.08 g/l
Buffers	1.7 g/l
Agar	158 g/l
pH	6.9

2.4.6 Catalase test

At times, the incomplete reduction of oxygen leads to the production of hydrogen peroxide (H_2O_2) or the superoxide free radical (O_2^-) that are both toxic to the cell. Many aerobic or facultatively anaerobic cells produce enzymes that remove these toxic byproducts. Superoxide dismutase removes the superoxide free radical in the following reaction ($2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2$). Catalase removes hydrogen peroxide in the following reaction ($2H_2O_2 \rightarrow 2H_2O + O_2$). Strict anaerobes usually lack these enzymes and therefore are restricted to an oxygen free environment. As only a subset of all the bacterial species produce cytochrome oxidase and catalase, one can test for the presence of these enzymes in bacteria to determine the oxygen tolerance of an individual bacterium and also to identify the bacterium.

Procedure: On a clean and labeled microscope slide using a sterile loop a small amount of culture from the plate was transferred. One drop of H_2O_2 solution was added to each culture sample and watch for the production of bubbles in the suspension. The bubbles result from oxygen gas that was released as a result of catalase activity that has converted the externally supplied hydrogen peroxide into oxygen and water. If no bubbles were produced the test was noted negative for catalase activity.

2.4.7 Cytochrome-oxidase test

Respiratory metabolism results in the conversion of glucose into energy in the form of ATP by the processes of glycolysis, Kreb's cycle and oxidative phosphorylation. In the process of respiratory metabolism, most of the ATP is generated as a result of the activity of the electron transport system. The electron transport system is composed of membrane associated molecules (cytochromes or flavoproteins) that accept and donate electrons. Reduced NADH and FADH from glycolysis and Kreb's cycle enter at or near the top of the electron transport chain and reduce the first molecule in the chain as they are concurrently re-oxidized. As electrons are passed down the chain of the electron transport system, each molecule in the chain alternate between reduced and oxidized forms. Cytochrome oxidase is found at the bottom of this chain, cytochrome oxidase catalyzes the re-oxidation of the last cytochrome molecule in the chain by molecular oxygen. This final process results in the reduction of molecular oxygen to water. The cytochrome-oxidase pathway is described in Figure 2.4.

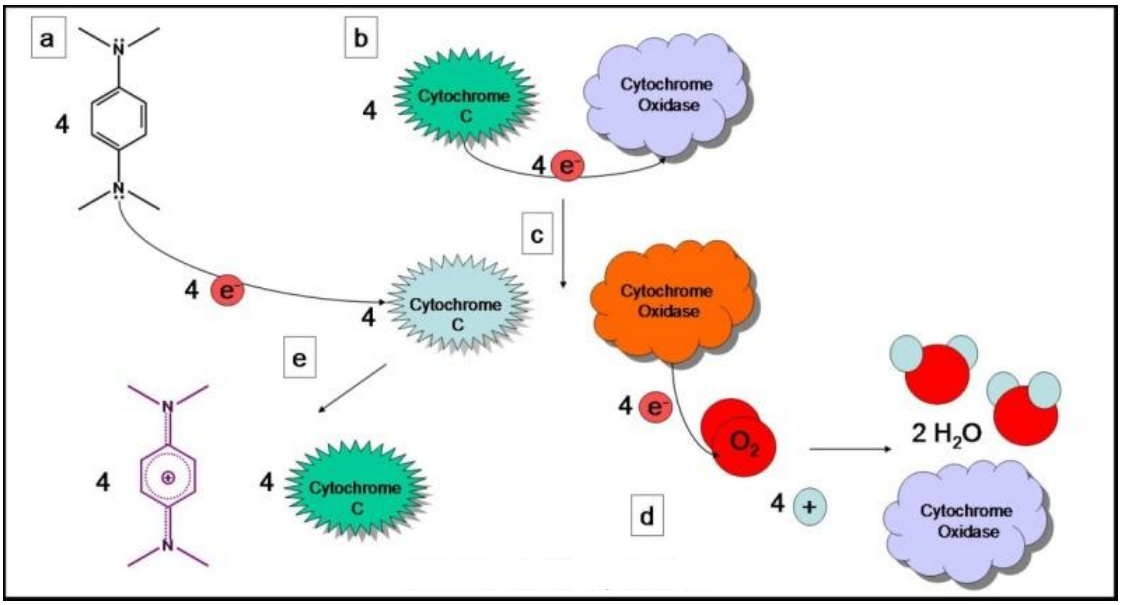


Figure 2.4 Cytochrome-Oxidase pathway (source: ASM MicrobeLibrary).

Procedure: On a labeled clean piece of filter paper using a sterile loop, a small amount of culture was transferred from the plate to the microscope slide. With a sterile wooden applicator the sample was rubbed into the paper without tearing it. This acts to remove capsule (glycocalx, mucin) from the surface of the bacteria so the substrate can interact with the bacteria. One drop of N, N, N′, N′-tetramethyl-p-phenylenediamine (TMPD) was added to each sample and the results were recorded immediately after 20 seconds.

TMPD is a chromogenic reducing reagent. In its reduced state TMPD is colorless; in its oxidized state it is deep purple or blue. Therefore, if the bacteria produce cytochrome oxidase, the reagent will be oxidized and will turn blue. The reaction is described below.

[Tetramethyl-p-phenylenediamine (reduced) + 2 Cytochrome oxidase (oxidized) → Tetramethyl-p-phenylenediamine (oxidized) + 2 Cytochrome oxidase (reduced)]

In a negative test for cytochrome oxidase the sample will remain clear, turn yellow or light grey.

2.4.8 Oxidative-fermentative (O-F) test

Bacteria produce acid from carbohydrates by two methods. One is an anaerobic process called fermentation; the second, designated oxidation, is an aerobic process. Since Gram negative bacteria which attack carbohydrates usually do so exclusively by either fermentation or oxidation, the determination of the type of carbohydrate metabolism carried out by an organism is of taxonomic significance.

Enteric fermentation broth carbohydrate media will detect acid production by fermentative organisms. Oxidative organisms do not usually produce a detectable amount of acid in enteric fermentation broth media since the acidity produced by oxidation is less than by fermentation and because sufficient alkaline products are frequently produced from the peptone in the medium to neutralize the acid produced by oxidative metabolism. Triple sugar iron agar (TSI) can be used to differentiate fermentative from oxidative bacteria. A TSI reaction of acid butt with or without an acid slant is indicative of a fermenter. No change of the butt and slant of TSI or slight alkalinization of the slant is indicative of an oxidizer or non-utilizer of sugars. Occasionally, an oxidizer will show slight acidity on the slant of TSI. In cases where the TSI reaction is doubtful, an oxidation-fermentation test can be carried out using O-F medium with 1% glucose (dextrose) as described.

An O-F medium was used to determine the pattern of carbohydrate metabolism of oxidative bacteria. O-F base medium contains less peptone than do the fermentation bases and contains 0.3% agar. The agar prevents convection currents and allows the acid produced to remain concentrated in the medium adjacent to where it is produced and not be diluted by mixing throughout the medium.

Two tubes of O-F glucose medium are used, one of which is covered with a layer of sterile mineral oil as an oxygen barrier. If glucose is metabolized in either tube, acidic end products accumulate and the pH drops. A pH indicator in the medium changes color to indicate acid production.

O-F glucose medium with oil overlay was used. This consists of a nutrient medium to which 1% glucose has been added. Mineral oil lighter in density that the medium was added to provide an airtight barrier. The pH indicator was bromothymol blue, which is green at neutral pH and turns yellow at pH <6.0. It gets its name because it turns blue at pH >7.6.

An inoculum from a pure culture was transferred aseptically to a tube of O-F glucose medium with oil overlay. The agar was stabbed through the oil to “plant” the bacteria in the butt of the tube. The inoculated tube was incubated at 35-37°C for 24 hours. A positive result for fermentation of glucose was indicated by the medium turning yellow.

O-F glucose is a semisolid medium incorporating a pH indicator, 1% glucose, and peptones. The type of metabolism a bacterium has aerobic use of a sugar vs. anaerobic use of a sugar; determines the color results in the two tubes used. The overlay of oil or vaspar wax prevents oxygen from getting into the medium of one tube: the other tube has air inside. Aerobic use of sugar is oxidative metabolism, anaerobic use is fermentative. Bacteria that oxidize carbohydrates are usually obligate aerobes, whereas those that ferment are usually facultative anaerobes. The fermentation will produce far more acid than the aerobic oxidation, but the O-F glucose test is very sensitive to the small amounts of acid produced via aerobic means. As the organism grows and uses the sugar, the resulting acid will change the pH, and the pH indicator will change colors. The indicator is bromothymol blue, starting as a forest green color at a neutral pH, and ending as a yellow with low pH and a blue with high pH. Using two tubes allows the identification of whether the organism is oxidative (only open tube acidic) or fermentative (both tubes acidic). If the organism uses the peptones rather than the glucose, the pH will become basic.

Procedure

- Two tubes were used for the O-F test.
- A generous amount of the test organism was transferred to the O-F medium by stabbing four (4) times approximately $^1/_3$ the depth of the medium.
- By using a straight inoculating needle two tubes were inoculated with each isolate.
- One inoculated tube from each pair was overlaid with 1 ml of mineral oil. (The tubes were overlaid with oil are called "closed" tubes). This overlay prevents the diffusion of oxygen into the medium and creates an anaerobic condition in the tube.
- Tubes were incubated with loose caps at 35°C for 24 hours.
- Following incubation, interpret the results based on the interpretive table below.

Interpretation of findings:

Open tube	Closed tube	Method of glucose utilization
Yellow (A)	Yellow (A)	Fermentative
Yellow (A)	Blue-Green (-)	Oxidative
Blue-Green (-)	Blue-Green (-)	Non-utilizer or possible no growth

Note: (A) = acid formation, (-) = no growth

2.4.9 Growth on Triple Sugar Iron (TSI) agar

TSI medium is a differential medium that can distinguish between a number of Gram-negative enteric bacteria based on their physiological ability (or lack thereof) to:

a. metabolize lactose and/or sucrose,
b. conduct fermentation to produce acid,
c. produce gas during fermentation, and
d. generate H_2S.

The medium contains 1.0% each of sucrose and lactose and 0.1% glucose. If only glucose is fermented, acid produced in the butt will turn it yellow, but insufficient acid products are formed to affect the methyl red in the slant. However, if either sucrose or lactose are fermented, sufficient fermentation products will be formed to turn both the butt and the slant yellow. If gas is formed during the fermentation, it will show in the butt either as

bubbles or as cracking of the agar. If no fermentation occurs (as for an obligate aerobe), the slant and butt will remain red. The medium also contains ferrous sulfate. If the bacterium forms H_2S, this chemical will react with the iron to form ferrous sulfide, which is seen as a black precipitate in the butt (a black butt).

Procedure: Add 3.0 g of beef extract, 3.0 g of yeast extract, 15.0 g of peptone, 5.0 g of protease peptone, 10.0 g of lactose, 10.0 g of saccharose, 1.0 g of glucose, 0.2 g of ferrous sulphate, 5.0 g of sodium chloride, 0.3 g of sodium thiosulphate, 0.024 g of phenol red and 12.0 g of agar and make the mixture up to 1000 ml with distilled water. The peptone mixture and the beef and yeast extracts provide the nutrients essential for growth. Sodium chloride maintains the osmotic balance of the medium. The bacteriological agar is the solidifying agent. In another replication TSI media from Hi-media, India was used and results were the same. Following steps were carried out in both the cases:

- Sterilize the inoculating needle till red hot and then allowed to cool.
- LB agar plate containing the 24-48 hour culture was taken.
- Using aseptic technique, the culture of the organism was taken from LB plate with the needle.
- A sterile TSI slant tube from the rack was taken, the cap was removed and flame the neck of the tube.
- Stab the needle containing the pure culture into the medium, up to the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant.
- Again flamed the neck of the TSI tube, capped and placed it in the test tube rack.
- Incubated at 37^oC for 18 to 24 hours.

Interpretation of findings: When TSI agar is inoculated with a glucose-only fermenter, acid products lower the pH and turn the entire medium yellow within a few hours. Because glucose is in short supply (0.1%), it will be exhausted within about 12 hours. As the glucose is used up, the organisms located in the aerobic region (slant) will begin to break down available amino acids, producing NH_3 and raising the pH. This process, which takes 18 to 24 hours to complete, is called a reversion and only occurs in the slant because of the anaerobic conditions in the butt. Thus, a TSI agar with a red slant and yellow butt after a 24 hour incubation period indicates that the organism ferments glucose but not lactose.

Organisms that are able to ferment glucose and lactose and/or sucrose also turn the medium yellow throughout. However, because the lactose and sucrose concentrations are ten times higher than that of glucose, greater acid production results and both slant and butt will remain yellow after 24 hours. Therefore, a TSI agar with a yellow slant and butt at 24 hours indicates that the organism ferments glucose and one or both of the other sugars. Gas produced by carbohydrate fermentation will appear as fissures in the medium or will lift the agar off the bottom of the tube.

Hydrogen sulfide (H_2S) may be produced by the reduction of thiosulfate in the medium or by the breakdown of cysteine in the peptone. Ferrous sulfate in the medium reacts with the H_2S to form a black precipitate, usually seen in the butt. Acid conditions must exist for thiosulfate reduction; therefore, black precipitate in the medium is an indication of sulfur reduction and fermentation. If the black precipitate obscures the color of the butt, the color of the slant determines which carbohydrates have been fermented (i.e., red slant = glucose fermentation, yellow slant = glucose and lactose and/or sucrose fermentation).

	Result (slant/butt)	Symbol	Interpretation
1	Red/Yellow	K/A	Glucose fermentation only, peptone catabolized.
2	Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation.
3	Red/Red	K/K	No fermentation, Peptone catabolized.
4	Yellow/Yellow with bubbles	A/A, G	Glucose and lactose and/or sucrose fermentation, Gas produced.
5	Red/Yellow with bubbles	K/A, G	Glucose fermentation only, Gas produced.
6	Red/Yellow with bubbles and black precipitate	K/A, G, H_2S	Glucose fermentation only, Gas produced, H_2S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A, G, H_2S	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
8	Red/Yellow with black precipitate	K/A, H_2S	Glucose fermentation only, H_2S produced.
9	Yellow/Yellow with black precipitate	A/A, H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced.

Note: A = acid production, K = alkaline reaction, G = gas production

2.4.10 Growth on Sulfur Indole Motility (SIM) media (semisolid medium)

A medium for the differentiation of enteric bacteria on the basis of hydrogen sulphide (S) reduction and indole (I) production and motility (M). It tests the ability of an organism to do several things: reduce sulfur, produce indole and swim through the agar (be motile). SIM is commonly used to differentiate members of *Enterobacteriaceae*. Sulfur can be reduced to H_2S (hydrogen sulfide) either by catabolism of the amino acid cysteine by the enzyme cysteine desulfurase or by reduction of thiosulfate in anaerobic respiration. If hydrogen sulfide is produced, a black color forms in the medium. Bacteria that have the enzyme tryptophanase, can convert the amino acid, tryptophane to indole. Indole reacts with added Kovac's reagent to form rosindole dye which is red in color (indole +). SIM tubes were inoculated with a single stab to the bottom of the tube. If an organism is motile than the growth will radiate from the stab mark and make the entire tube appear turbid.

Formulation of medium:

Peptones	26.0 g/l
Sodium thiosulphate	0.2 g/l
Ferrous ammonium sulphate	0.2 g/l
Agar	3.5 g/l
pH	6.7

2.4.11 Urease (urea hydrolysis) and Motility Indole Urea (MIU) test

Testing the ability to produce urease, an enzyme that hydrolyze urea providing a source of nitrogen for bacteria. MIU test checks simultaneously for the production of indole, urea hydrolysis and bacteria motility. Medium, together with TSI and Simmons Citrate agar, is most used for *Enterobacteriaceae* identification, especially *Escherichia* and *Salmonella*.

Media preparation: Firstly, MIU base was prepared with

Proteose peptone	10 g
Beef extract	5 g
NaCl	5 g
KH_2PO_4	2 g
Phenol red solution $^1/_{500}$	6 ml
Agar	3 g
Distilled water	900 ml
pH	6.8-6.9

Secondly, urea-glucose solution was made with 10 g urea + 1 g glucose + 100 ml distilled water.

Preparation: To 90 ml MIU base medium 10 ml urea-glucose solution was added (base medium was melted by boiling it and wait to cool under 50°C before adding urea-glucose solution, otherwise urea will be inactivated. Final medium state was semi-solid).

Procedure: A well isolated colony was harvested and inoculated a MIU tube by stabbing the medium and incubated at 37°C for 24 hours.

Interpretation of findings: Motility is positive if the entire medium became opaque (semi-solid state of the medium permits the bacteria moving). Result is negative if the culture grows only on the stabbing line. For the indole test 2-3 drops of Ehrlich-Kovac's reactive wre added. A red layer on the surface of the medium appears if the reaction is positive. Urea is hydrolysed if the entire medium turns red.

2.4.12 Motility- hanging drop method

Many bacteria show no motion and are termed nonmotile. However, in an aqueous environment, these same bacteria appear to be moving erratically. This erratic movement is due to Brownian movement. Brownian movement results from the random motion of the water molecules bombarding the bacteria and causing them to move. True motility (self-propulsion) has been recognized in other bacteria and involves several different mechanisms. Bacteria that possess flagella exhibit flagellar motion. Helical-shaped spirochaetes have axial fibrils (modified flagella that wrap around the bacterium) that form axial filaments. These spirochaetes move in a corkscrew- and bending-type motion. Other bacteria simply slide over moist surfaces in a form of gliding motion. The above types of motility or nonmotility can be observed over a long period in a hanging drop slide (Figure 2.5). Hanging drop slides are also useful in observing the general shape of living bacteria and the arrangement of bacterial cells when they associate together. A ring of Vaseline around the edge of the coverslip keeps the slide from drying out.

Procedure

- With a toothpick, a small ring of petroleum jelly was spreaded around the concavity of a depression slide.

- After thoroughly mixing one of the cultures, using the inoculating loop to aseptically a small drop of one of the bacterial suspensions was placed in the center of a cover glass.
- The depression slide was lowered, with the concavity facing down, onto the cover glass so that the drop protrudes into the center of the concavity of the slide. Press gently to form a seal.
- Turned the hanging drop slide over and placed on the stage of the microscope so that the drop was over the light hole.
- The drop was examined by first locating its edge under low power and focusing on the drop. Switch to the high-dry objective and then, using immersion oil, to the 100× objective. In order to see the bacteria clearly, the diaphragm was closed as much as possible for increased contrast. Bacterial shape, size, arrangement, and motility was noted.

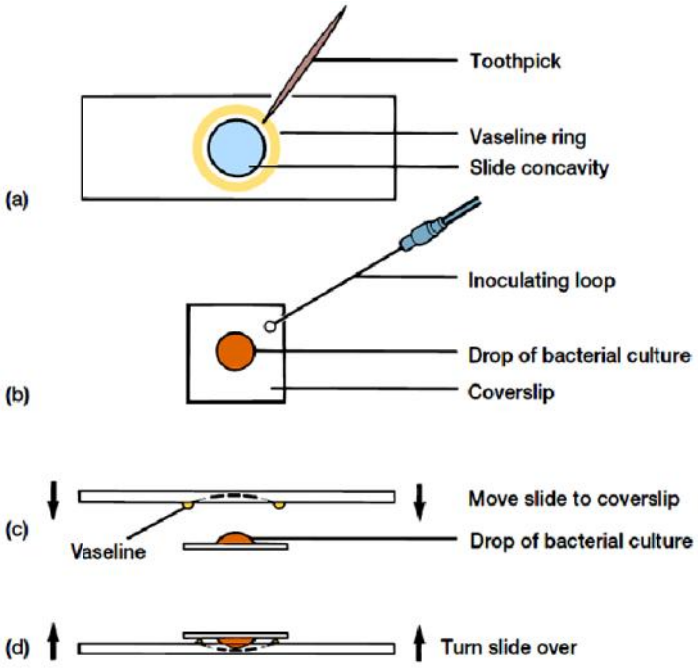


Figure 2.5 Preparation of a hanging drop slide (source: Harley-Prescott, Laboratory exercises in microbiology, 2002).

2.4.13 Methyl red test and Voges-Proskauer reaction

These tests both use the same broth for bacterial growth. The broth is called MRVP broth. After growth, the broth was separated into two different tubes, one for the methyl red (MR) test and one for the Voges-Proskauer (VP) reaction.

The methyl red test detects production of acids formed during metabolism using mixed acid fermentation pathway using pyruvate as a substrate. The pH indicator Methyl Red was added to one tube and a red color appears at pH's lower than 4.2, indicating a positive test (mixed acid fermentation was used). The solution remaining yellow (pH = 6.2 or above) indicates a negative test, meaning the butanediol fermentation was used.

The VP reaction uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2,3-butanediol fermentation pathway. After adding both reagents, the tube was shaken vigorously then allowed to sit for 5-10 minutes. A pinkish-red color indicates a positive test, meaning the 2,3-butanediol fermentation pathway was used.

Media preparation: Separately 5 g of glucose, 5 g of peptone and 5 g of dipotassium hydrogen phosphate was weighed and all the ingredients were suspended in distilled water. Made up to 1000 ml. pH was adjusted to 6.9. Three (3) ml of the media was dispensed into each test tube, which were plugged and sterilized at 121°C.

Reagents needed: Barritt's reagents A and B

Preparation of Barritt's reagent: It consists of two solutions;

1. Solution A was prepared by dissolving 6 grams of α-naphtholin in 100 ml of 95% ethyl alcohol.
2. Solution B was prepared by dissolving 16 grams of potassium hydroxide in 100 ml of water.

Procedure: Using sterile technique, each experimental organism was inoculated to the appropriately labeled tube of medium by means of loop inoculation. The cultures were incubated for 24-48 hours at 37°C. The experiment should be conducted in the Laminar Air Flow (LAF). Arrange the materials required for the experiment in the LAF.

- From an 18-24 hour old culture the tube was lightly inoculated by a single colony.
- The tubes were incubated at 35-37°C for 48 hours with slightly loosen cap.
- After incubation, using a sterile pipette to remove two - 1ml aliquots and place into two small tubes (one tube was for the methyl red test and the other for the Voges-Proskauer reaction).
- 5 drops of methyl red was added to one tube. The results were noted immediately.

- For the Voges-Proskauer reaction 15 drops of Voges-Proskauer reagent A was added. Oxygen was needed to complete the reaction, so to aerate the sample mixed them well.
- 5 drops of Voges-Proskauer reagent B was added to the tube and mixed well to aerate the sample.

Interpretation of findings

Methyl Red - A red color at the surface was considered a positive result. A negative test was indicated by a yellow color at the surface.

Voges-Proskauer - A positive test was indicated by a pink-red color developing within 5 minutes.

MR positive result:

Glucose ------Glucose Metabolism-------> Pyruvic Acid

Pyruvic acid ----------> Acetoin

Acetoin + added alpha-naphthol + added KOH = red color

MR negative result:

Glucose ------Glucose Metabolism-------> Pyruvic Acid

Pyruvic acid ----------> No Acetoin

No acetoin + added alpha-naphthol + added KOH = copper color

2.4.14 Acid production from sugars

(carbohydrate/sugar utilization/fermentation test)

This test was performed to determine the ability of microbes to ferment carbohydrates with the production of an acid and/or gas.

Principle: Sugars are metabolized through different metabolic pathways depending on types of microbial species and aerobic or anaerobic environment. If fermenting bacteria are grown in a liquid culture medium containing the carbohydrate, they may produce organic acids as by-products of the fermentation. These acids are released into the medium and so lower pH of medium. If a pH indicator such as phenol red or bromocresol blue is included in the medium, the acid production will change the medium from its original color to yellow. Gases produced during the fermentation process can be detected by using a small, inverted tube, called a Durham tube, within the liquid culture medium. If gas is produced, the liquid medium inside the Durham tube

will be replaced by the gas in the form of a bubble. Phenol Red Broth Base was used as a basal medium to which carbohydrates were added for determination of fermentation reactions of pure cultures of microorganisms.

Procedure: The ingredients (peptone, NaCl, Beef extract and phenol red) were suspended into distilled water, mixed well. To ensure complete solution heated and 5 ml of media was distributed in fermentation tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 30 minutes and filter sterilized carbohydrate solution or carbohydrate disc was aseptically added to sterile basal medium. The tubes were incubated at 37ºC for 24-36 hours.

Stock solution:

(i) Using carbohydrate stock solution: Dilute 2 g of carbohydrate in 10 ml distilled water. Then filtered the solution and 300 µl of stock solution was added to each tube (5 ml) to reach the concentration at 5-10%.

(ii) Using carbohydrate disc: The carbohydrate disc (25 mg/disc) was dispensed in 5 ml phenol red broth solution.

The result was recorded as heavy growth (++, gives opaque solution), growth (+, look turbid) and no growth (-, gives a transparent or clear look as control tube). This test was performed with carbohydrate disc (Hi-media, India) also. The phenol red glucose broth was inoculated with bacteria into the tube with the disc and incubated at 37°C.

Interpretation of findings: If the medium changes from colorless to yellow and gas bubble was found in Durham's tube then it indicates acid and gas production. In some cases gas may not be evolved during the process. If no change observed in the color of medium then sugar was not degraded by the organism.

2.4.15 Antibiotic susceptibility test

Bacteria demonstrate two kinds of resistance to antibiotics, namely intrinsic resistance and acquired resistance. Intrinsic resistance means that the species was resistant to an antibiotic even before its introduction. Acquired resistance means that the species was originally susceptible to an antibiotic, but later became resistant. Bacteria can acquire antibiotic resistance either by mutation or through exchange of genetic material among same or closely related species. The sudden acquisition of resistance to antibiotics poses

difficulties in treating infections. Resistance to several different antibiotics at the same time is even more significant problem. It is because of the acquired resistance that bacterial isolates must be subjected to antibiotic susceptibility testing.

When fish become exposed to antimicrobial compounds, there will undoubtedly be an impact on the composition of the microflora and on antibiotic resistance patterns (Austin and Al-Zahrani 1988, Hansen *et al*. 1992, Alvarez *et al*. 2001, Pedersen and Dalsgaard 2003, Cabello 2004, Moffitt and Mobin 2006). This, in turn, may impact upon the transmission of antibiotic resistance, such as via R-factors (Alvarez *et al*. 2001), to other bacteria, and perhaps of significance to humans (Austin 2006).

Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. Resistance factors, particularly those carried on mobile elements, can spread rapidly within human and animal populations. Multidrug-resistant pathogens travel not only locally but also globally, with newly introduced pathogens spreading rapidly in susceptible hosts. Antibiotic resistance patterns may vary locally and regionally, so surveillance data needs to be collected from selected sentinel sources. Patterns can change rapidly and they need to be monitored closely because of their implications for public health and as an indicator of appropriate or inappropriate antibiotic usage by physicians in that area.

Lalitha (2004) stated that with the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper disc is by far the commonest type used. The disc diffusion method of antimicrobial susceptibility testing (AST) is the most practical method and is still the method of choice for the average laboratory. Automation may force the method out of the diagnostic laboratory but in this country as well as in the smaller laboratories of even advanced countries, it will certainly be the most commonly carried out microbiological test for many years to come. It is, therefore, imperative that microbiologists understand the principles of the test well and keep updating the

information as and when necessary. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth. Even automated techniques are variations of the above methods.

Antimicrobial resistance is a global problem. Emergence of multidrug resistance has limited the therapeutic options; hence monitoring resistance is of paramount importance. Antimicrobial resistance monitoring will help to review the current status of antimicrobial resistance locally, nationally and globally and helpful in minimizing the consequence of drug resistance, limit the emergence and spread of drug resistant pathogens.

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include as diffusion, dilution, and diffusion and dilution.

Diffusion	Dilution	Diffusion & Dilution
Stokes method	Minimum Inhibitory Concentration	E-Test method
Kirby-Bauer method	i) Broth dilution ii) Agar Dilution	

A wide range of different β-lactam antibiotic discs were used to determine the resistance and sensitivity pattern of the isolates. The isolated bacterial strains were grown overnight in nutrient broths that were placed in the orbital shaker at 30°C temperature at 160 rpm. Fresh nutrient broth cultures of 18 to 20 hours old were spreaded on to previously made nutrient agar plate. After soaking, antibiotic discs from different manufacturer (Oxoid Ltd., England; Becton-Dickinson, USA; Mast Diagnostics, UK) were placed and inverted petri plates were kept in incubator for overnight incubation at ~37°C. The diameter of the inhibition zone was measured (mm). Gentamicin, Neomycin, Azithromycin, Erythromycin, Ampicillin, Cephradine, Doxycycline, Tetracycline, Ceftriaxone, Ciprofloxacin, Pefloxacin, Mecillinam, Sulphamethoxazole/ Trimethoprim, Nitrofurantoin and Bacitracin discs were used in the present study.

2.4.16 Colony forming unit (CFU) determination

In microbiology, colony forming unit (CFU) is a measure of viable bacterial or fungal numbers. Unlike in direct microscopic counts where all cells, dead and living, are

counted, CFU measures viable cells. By convenience the results are given as CFU/ml, colony forming units per milliliter.

The theory behind the technique of CFU establish that a single bacterium can grow and become a colony, via binary fission. These colonies are clearly different between each other, both microscopical and macroscopical. However, some bacteria do not separate completely during the sample preparation process (*Staphylococcus*, *Streptococcus*) and the results of the count will be below the number of individual cells using direct methods.

A colony is a cluster of microorganisms growing on the surface of or within a solid medium; usually cultured from a single cell. Each bacterial cell arises either by division of a preexisting cell with similar characteristics (i.e., binary fission), or through a combination of elements from two such cells in a sexual process.

Statistical significance, when applied to incubated plates, depends upon the research hypothesis, the power of the test, the difference between the numbers of colonies observed under the experimental and control conditions, and the accuracy of the counting methodology.

Many studies require the quantitative determination of bacterial populations. The two most widely used methods for determining bacterial numbers are the standard, or viable, plate count method and spectrophotometric (turbidimetric) analysis. Although the two methods are somewhat similar in the results they yield, there are distinct differences. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive.

The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 25 and 250 colonies. Fewer than 25 colonies are not acceptable for statistical reasons, and more than 250 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony forming units (CFUs).

The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of live bacteria that can grow under the incubation conditions employed. A wide series of dilutions was normally plated because the exact number of live bacteria in the sample was usually unknown. Greater precision is achieved by plating duplicates or triplicates of each dilution. Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, it was observed that the amount of transmitted light decreases as the cell population increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^7 cells or greater.

Procedure

Standard plate count (Figure 2.6)

- The bottoms of six petri plates were labeled with the following dilutions: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. Four bottles of saline or phosphate buffer solution were labeled as 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}.

- Using aseptic technique, the initial dilution was made by transferring 1.0 ml of liquid sample to a 99.0 ml sterile saline blank. This is a $^1/_{100}$ or 10^{-2} dilution. The bottle was capped.

- The 10^{-2} blank was then shaken vigorously 25 times by placing one's elbow on the bench and moving the forearm rapidly in an arc from the bench surface and back. This serves to distribute the bacteria and break up any clumps of bacteria that may be present.

- Immediately after the 10^{-2} blank has been shaken, uncapped it and aseptically transferred 1.0 ml to a second 99 ml saline blank. Since this was a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample. The bottle was capped.

- Shake the 10^{-4} blank vigorously 25 times and transferred 1.0 ml to the third 99 ml blank. This third blank represents a 10^{-6} dilution of the original sample. The process was repeated once more to produce a 10^{-8} dilution.

- Shake the 10^{-4} blank again and aseptically transfer 1.0 ml to one petri plate and 0.1 ml to another petri plate. The same was done for the 10^{-6} and the 10^{-8} blanks.

- Remove one agar pour tube from the 48° to 50°C water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically poured the agar into it. The agar and sample were immediately mixed by gently moving the plate in a figure-eight motion while it rests on the tabletop. This process repeated for the remaining five plates.

- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 35°C for 24 hours.

- At the end of the incubation period, all of the petri plates containing between 25 and 250 colonies were selected for counting. Plates with more than 250 colonies cannot be counted and were designated too numerous to count (TNTC). Plates with fewer than 25 colonies were designated too few to count (TFTC). Count the colonies on each plate.

- The number of bacteria (CFU) per milliliter or gram of sample was calculated by dividing the number of colonies by the dilution factor. The number of colonies per ml reported should reflect the precision of the method and should not include more than two significant figures.

2.4.17 Minimum Inhibitory Concentration (MIC)
(broth tube dilution method)

A current definition of the Minimum Inhibitory Concentration, MIC, is "the lowest concentration which resulted in maintenance or reduction of inoculums viability" (Carson *et al*. 1995). Minimum inhibitory concentration is a difficult subject. The traditional methods of analysis do not allow a quantitative measurement; MIC is, in effect, a range of concentrations depending on the dilution series used. Measurement of the MIC should be a first line technique of discovery for the biocide and preservative scientist, but in many laboratories, it is used as an indicator of activity rather than something of real substantive value (Lambert and Pearson 2000).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (MBC) testing define a test material's potency in terms of the concentration at which it will inhibit growth (MIC) or completely kill (MBC) microorganisms during a 18 to 20 hour period of incubated (35 ± 2°C) exposure. It means the lowest concentration of antibiotic that completely prevented bacterium growth or completely kill them. MIC and MBC test was done by broth tube dilution method (Figure 2.7, 2.8) which is as follows:

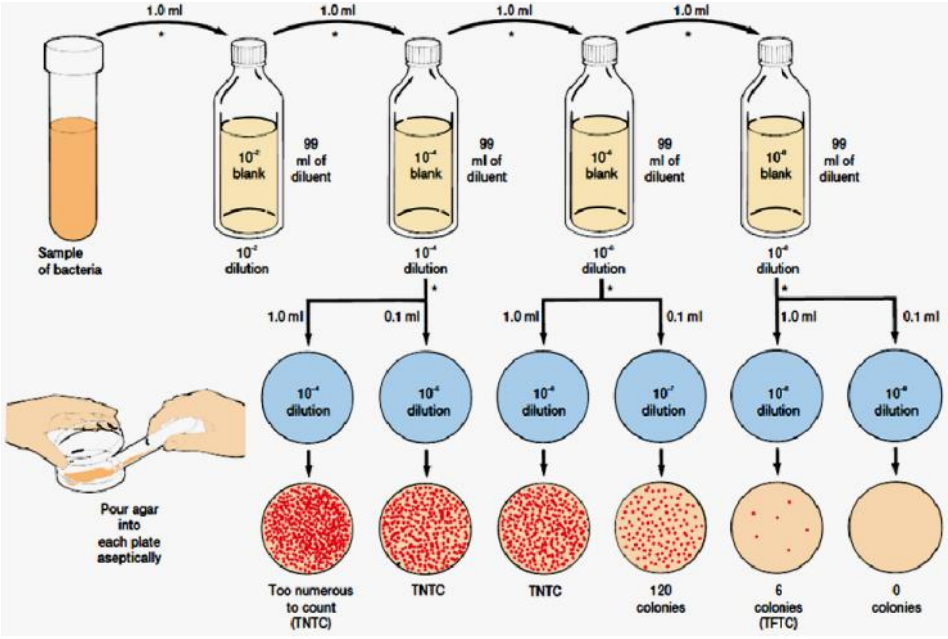


Figure 2.6 Determination of CFU by standard plate count method (source: Harley-Prescott, Laboratory exercises in microbiology, 2002).

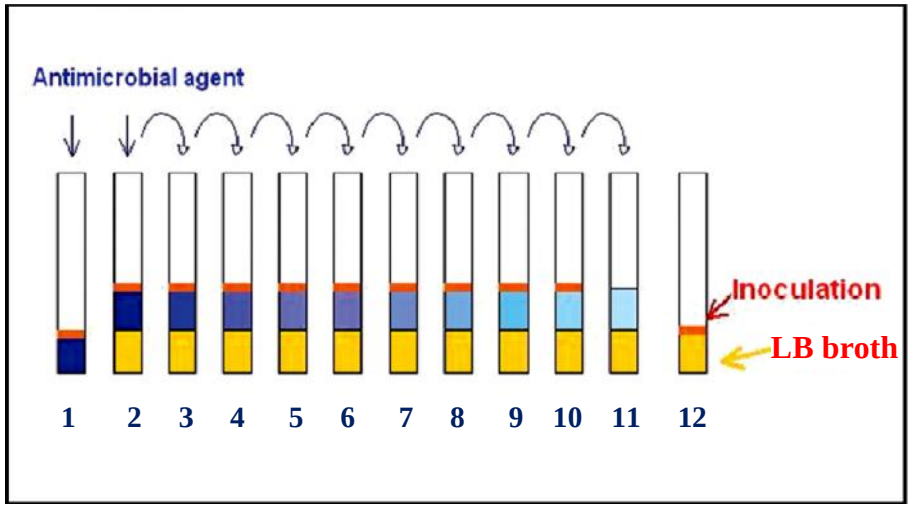


Figure 2.7 MIC broth tube dilution method.

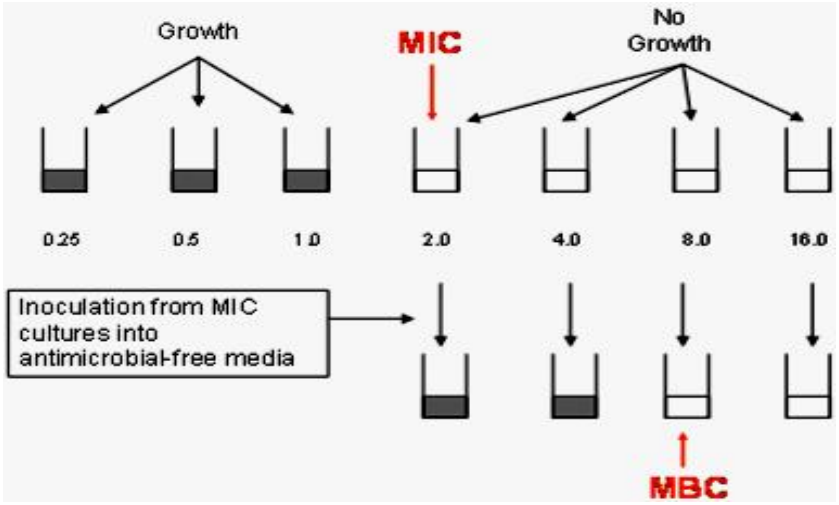


Figure 2.8 MBC broth tube dilution method.

The tube dilution test is the standard method for determining levels of resistance to an antibiotic. Serial dilutions of the antibiotic are made in a liquid medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity is considered to be the MIC. At this dilution the antibiotic is bacteriostatic. Additionally, the MBC was determined by subculturing the contents of the tubes onto antibiotic-free solid medium and examining for bacterial growth.

Although the tube dilution test is fairly precise, the test is laborious because serial dilutions of the antibiotic must be made and only one isolate can be tested in each series of dilutions.

Procedure

- Sterile capped test tubes were numbered 1 through 9. All of the following steps are carried out using aseptic technique.
- 2.0 ml of antibiotic solution (1280 µg/ml) was added to the first tube.
- 1.0 ml of sterile broth was added to all other tubes.
- 1.0 ml from the first tube was transferred to the second tube.
- Using a separate pipette, the contents of this tube were mixed and 1.0 ml was transferred to the third tube.
- Continue dilutions in this manner to tube number 8, being certain to change pipettes between tubes to prevent carryover of antibiotic on the external surface of the pipette.
- 1.0 ml from tube 8 was removed and discarded. The ninth tube, which serves as a control, receives no antibiotic.
- Suspend to an appropriate turbidity several colonies of the culture to be tested in 5.0 ml of nutrient broth to give a slightly turbid suspension.
- This suspension was diluted by aseptically pipetting 0.2 ml of the suspension into 40 ml of nutrient broth.
- 1.0 ml of the diluted culture suspension was added to each of the tubes. The final concentration of antibiotic is now one-half of the original concentration in each tube.

- All tubes were incubated at 35^{o}C overnight.
- Tubes for visible signs of bacterial growth were examined. The highest dilution without growth is the minimum inhibitory concentration (MIC).
- MBC was determined from broth dilution minimum inhibitory concentration (MIC) tested by subculturing 1 ml of culture to 1 ml nutrient broth.

Minimum inhibitory concentration was determined when an organism does not respond to treatment thought to be adequate, relapses while being treated or when there is immunosuppression. Broth dilution testing allows the option of providing both quantitative (MIC) and qualitative (category interpretation) results. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Preparation of antibiotic stock solution: Antibiotic stock solution was prepared by commercially available antimicrobial powders (with given potency) and the amount needed and the diluents in which it can be dissolved can be calculated by using either of the following formulas to determine the amount of powder (1) or diluent (2) needed for a standard solution:

$$\text{Weight mg} = \frac{\text{Volume ml} \times \text{Concentration (µg/ml)}}{\text{Potency (µg/mg)}}$$

or

$$\text{Volume ml} = \frac{\text{Weight mg} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/ml)}}$$

Antimicrobial agent stock solutions was prepared at concentrations of at least 1000 µg/ml (example: 1280 µg/ml) or 10 times the highest concentration to be tested, whichever was greater. Solutions were sterilized by membrane filtration. Small volumes of the sterile stock solutions were dispensed into sterile glass vials; carefully sealed; and stored.

Preparation of antibiotic dilution range:

- Sterile 13×100 mm test tubes were used to conduct the test.
- The tubes were closed with loose screw-caps, or cotton plugs.
- The final twofold dilutions of antimicrobial agent were prepared volumetrically in the broth. A minimum final volume of 1 ml of each dilution was used for the test.

Preparation of inoculums:

- The inoculums were prepared by making a direct broth suspension of isolated colonies selected from an 18 to 24 hour agar plate.
- Optimally within 15 minutes of preparation, the adjusted inoculum suspension was diluted in broth so, after inoculation, each tube contains approximately 5×10^5 CFU/ml. This can be accomplished by dilution resulting in a tube containing approximately 1×10^6 CFU/ml.

Inoculation: Within 15 minutes after the inoculums has been standardized as described above, 1 ml of the adjusted inoculum was added to each tube containing 1 ml of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and mix. This results in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculums. The inoculated tubes were incubated at 35±2ºC for 16 to 20 hours in an ambient air incubator. The same incubation temperature was maintained for all cultures.

Interpretation: The amount of growth were compared in the tubes containing the antimicrobial agent with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set of tests when determining the growth end points. The lowest concentration at which the isolate was completely inhibited (as evidenced by the absence of visible bacterial growth) was recorded as the minimal inhibitory concentration or MIC.

2.4.18 Optimization of the isolates

2.4.18.1 Effect of temperature on growth: Microorganisms have been found growing in virtually all environments where there is liquid water, regardless of its temperature. In 1966, Professor Thomas D. Brock at Indiana University, made the amazing discovery in boiling hot springs of Yellowstone National Park that bacteria were not just surviving there, they were growing and flourishing. Boiling temperature could not inactivate any essential enzyme. Subsequently, procaryotes have been detected growing around black smokers and hydrothermal vents in the deep sea at temperatures at least as high as 115°C. Microorganisms have been found growing at very low temperatures as well. In supercooled solutions of H_2O as low as -20°C, certain organisms can extract water for growth, and many forms of life flourish in the icy waters of the Antarctic, as well as household refrigerators, near 0°C.

Thermophiles are adapted to temperatures above 60°C in a variety of ways. Often thermophiles have a high G + C content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum temperature for growth. But this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means. The membrane fatty acids of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures. The membranes of hyperthermophiles, virtually all of which are Archaea, are not composed of fatty acids but of repeating subunits of the C5 compound, phytane, a branched, saturated, "isoprenoid" substance, which contributes heavily to the ability of these bacteria to live in superheated environments. The structural proteins, e.g. ribosomal proteins, transport proteins (permeases) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts. The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure, which accounts for their thermal stability.

2.4.18.2 Effect of pH on growth: The pH or hydrogen ion concentration [H^+], of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Appreciating that pH is measured on a logarithmic scale, the [H^+] of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes. Most free-living procaryotes can grow over a range of 3 pH units, about a thousand fold changes in [H^+]. The range of pH over which an organism grows is defined by three cardinal points: the minimum pH, below which the organism cannot grow, the maximum pH, above which the organism cannot grow, and the optimum pH, at which the organism grows best. For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing [H^+] on the rates of enzymatic reaction.

In the construction and use of culture media, we always consider the optimum pH for growth of a desired organism and incorporate buffers in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth. Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.

2.4.18.3 Effect of salinity on growth: Though microorganisms can grow at variant environment there is a optimum condition to grow. Marine bacteria needed high salt concentration for their growth, where bacteria living in freshwater environment do not need such salinity. The only common solute in nature that occurs over a wide concentration range is salt (NaCl), and microorganisms are named based on their growth response to salt. Microorganisms that require some NaCl for growth are halophiles. Mild halophiles require 1-6% salt, moderate halophiles require 6-15% salt, extreme halophiles require 15-30% NaCl for growth. Bacteria that are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl, are called halotolerant. Although halophiles are "osmophiles" (and halotolerant organisms are "osmotolerant") the term osmophiles is usually reserved for organisms that are able to live in environments high in sugar. Organisms which live in dry environments (made dry by lack of water) are called xerophiles.

In the present study there is an opportunity to record the tolerance limit of the isolates at different temperature (25, 29, 33 and 37°C), pH (6, 7 and 8) and salinity (1, 2 and 3%). In this purpose nutrient broth was prepared following manufacturer's instruction and kept at an orbital shaker where temperature was maintained in case of temperature optimization. This was perhaps the most laborious work done and no doubt a series of factors were maintained among all the protocol done in the present study. Procedures done in this purpose and reading of photoelectric colorimeter were recorded at purifying platform.

2.4.19 Molecular identification

The last fifteen years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology, following the cellular and protein era of the 1970s and 1980s. This explosion of technologies from the primary discipline of molecular biology has had major consequences and has allowed for significant developments in many areas of the life sciences, including bacteriology. Although, most modern clinical microbiology diagnostic laboratories rely on a combination of colonial morphology, physiology and biochemical/serological markers, for their successful identification either to the genus level or more frequently to the species level. It is important that organisms are correctly identified for a number of reasons, including the correct epidemiological reporting of causal agents in a given disease state, as well as for infection control purposes (Millar *et al*. 2007).

Molecular methods are increasingly being used. For example, numerous publications have discussed the sequencing of 16S rRNA genes (Haygood *et al*. 1992, Haygood 1993, van der Maarel *et al*. 1999, Spanggaard *et al*. 2000, Holben *et al*. 2002, Kim *et al*. 2007). Woo *et al*. (2008) gave a description and mentioned that with the use of 16S rDNA sequencing, 215 novel bacterial species, 29 of which belong to novel genera, have been discovered from human specimens in the past 7 years of the 21st century (2001–2007). Approaches have gone from the traditional (Liston 1957), through numerical taxonomy studies involving large numbers of isolates (e.g., 197 cultures investigated in one study (Grimes *et al*. 1993); 473 isolates studied in another (Montes *et al*. 1999)), to culture-independent molecular approaches (e.g., partial sequencing of the 16S rRNA gene (Spanggaard *et al*. 2000, Kim *et al*. 2007)). The benefit of the later is the recognition of organisms that may or may not be culturable by conventional techniques (Huber *et al*. 2004). Sometimes, the phenetic approach has centered on the use of rapid systems, such as BIOLOG or MIDI (Nedoluha and Westhoff 1997a, b). It is encouraging that some comparative studies have pointed to congruence between phenotypic and molecular analyses (Spanggaard *et al*. 2000). Overall, it would appear that narrow-based studies focusing on a limited number of bacterial groups are often more successful than those that attempt to be broad-based, trying to consider all of the bacteria from fish. From the published literature, it is apparent that there are many similarities between the bacterial populations in fish and water (Liston 1957, Evelyn and McDermott 1961, Colwell 1962, Pacha and Porter 1968, Simidu and Kaneko 1969, Austin 1982 and 1983, Allen *et al*. 1983b, Nieto *et al*. 1984, Apun *et* al. 1999, Diler *et al*. 2000). Bacterial taxonomy has progressed from reliance on highly artificial culture-dependent techniques involving the study of phenotype (including morphological, biochemical and physiological data) to the modern applications of molecular biology, most recently 16S rRNA gene sequencing, which gives an insight into evolutionary pathways (= phylogenetics) (Austin 2011).

There is evidence that molecular techniques have been used with increasing regularity for bacterial pathogens. A timely overview of PCR with emphasis on validation of the techniques and problems relating to diagnosis has been published (Hiney and Smith 1998). Molecular methods, namely sequencing of the 16S rRNA gene, permit the study of evolutionary relationships, i.e. phylogenetics, which may be viewed as phylogenetic trees, which are interpreted by cladistics and used in defining taxa (Austin 2011).

2.4.19.1 DNA-based diagnostics

Developments in bacterial ecology and industrial biotechnology are severely hampered by the lack of reliable identification system (Bull *et al*. 1993, Goodfellow and O'Donnell 1993). To obtain a phenotypic description requires long and fastidious work, which does not always warrant satisfactory identifications of bacterial species. Following determination of the structure of DNA by Watson and Crick (1953), studies in bacteriology have seen a major shift from functional to molecular techniques for identifying bacteria. Phylogenetic relationships among various organisms and their identification now can be derived from degree of DNA relatedness of their genomes: two closely related organisms share significant homologies, while distant organisms display low homologies. DNA-DNA relatedness is best suited for identification of closely related species or strains within a single species no doubt. Presently, a direct comparison of rRNA genes sequence is probably the most powerful tool for the identification of many bacteria (Stackebrandt and Goodfellow 1991). These genes have acquired paramount relevance for the study of bacterial evolution and phylogeny. Indeed, rRNA genes (rDNA) are present and expressed in all bacterial species, are truly homologous in all organisms, are easily sequenced and now offer a large and ever increasing database of sequences and allow the identification of bacteria (Amann *et al*. 1994). The sequences of gene coding for 16S rDNA is also a powerful tool for deducing phylogenetic and evolutionary relationships among eubacteria, archaebacteria and eucaryotic organisms because of their high information content, conservative nature and universal distribution (Lane *et al*. 1985, Woese 1987). The 16S rDNA sequence analysis is a standard method for the investigation of their phylogenetic relationships (Yamamoto and Harayama 1995). Even though some closely related species may have only a few differences in their 16S rDNA sequences, a phylogenetic tree can be established to give them an exact taxonomic position (Collins *et al*. 1991).

This study was therefore undertaken to sequence 16S rRNA gene of isolated bacteria identified in previous section and an attempt to: (i) clarify the intrageneric relationships of identified isolates and compare the sequence similarities with Genebank databases; (ii) to know the taxonomic position of the identified isolates; and (iii) identify regions in 16S rRNA genes which will have a value to develop probe in future.

The most recent advances in diagnostics have come from the field of Molecular Biology. The cloning and manipulating of genetic material has led to the development of extremely sensitive and specific diagnostic systems (Lipton *et al*. 1998). DNA based test formats have entered into the area of infectious disease diagnosis for aquatic species. Reddington and Lightner (1994) confirmed that the DNA probe is created by purifying the infectious agent of interest and isolating its nucleic acid. An exact copy of the DNA or a portion of the DNA is made by the cloning process. This copy or probe will bind to the original DNA of the pathogen whenever the two come into contact. In order to accomplish this efficiently, the DNA strands of both the pathogen and the probe must first be separated by heating. After the strands have been separated, one of the strands of the probe can bind to its complementary strand from the pathogen.

Molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish diseases and the epidemiology of bacterial, viral, and parasitical diseases. Molecular techniques are potentially faster and more sensitive than culture, serology, and histology methods that are traditionally used to identify fish pathogens. Pathogens can be detected from asymptomatic fish by molecular diagnostic techniques so disease outbreak could be prevented. Thus antibiotic treatment can be reduced so that creation of antibiotic resistant bacteria may be eliminated (Altinok and Kurt 2003).

Traditionally the diagnosis of the disease is carried out by agar cultivation and then phenotypic and serological properties of the pathogen or histological examination (Bernardet *et al*. 1990, Pazos *et al*. 1996). Furthermore, some of the bacteria could not be differentiated by conventional diagnostic methods from other phenotypically similar bacteria of the same genera (Shewan and McMeekin 1983). Some attempts have been made using biochemical tests, DNA homology and protease variability (Pyle and Shotts 1980, Bertolini and Rohovec 1992, Chen *et al*. 1995), but these techniques have some disadvantages such as need for previous isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen.

Genomic DNA isolation protocol

Cultures were grown to mid log-phase at 25°C in 100 ml nutrient broth in a orbital shaker (VS 201D, Vision Scientific, Korea). Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit (Tiangen, China) and purified

according to the manufacturer's instruction and used apparatus details were presented in Table 2.2 and Appendix III.

- 1-5 ml bacterial culture suspension was pipetted in a microcentrifuge tube by centrifuging for 1 min at 10,000 rpm (~11,500×g). Supernatant was discarded.

- 200 µl Buffer GA was added and mixed thoroughly by vortexing.

  For the more difficult broken Gram-positive bacteria step 2 was skipped, lysozyme was added, the specific methods were: 180 µl enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton® X-100; immediately before use, add lysozyme to 20 mg/ml) was added. Incubated for 30 min at 37°C.

  If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml, should be prepared by user, Cat. No.: RT405-11), mix by vortexing for 15s, and incubate for 5 min at room temperature (15–25°C).

- 20 µl Proteinase K was added and mixed thoroughly by vortexing.

- 220 µl Buffer GB was added to the sample, mixed thoroughly by vortexing, and incubated at 70°C for 10 min to yield a homogeneous solution. Briefly centrifuged the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

- 220 µl ethanol (96-100%) was added to the sample, and mixed thoroughly by vortexing for 15s. Briefly centrifuged the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

- The mixture was pipetted from step 5 into the TIANamp Spin Column CB3 (in a 2 ml collection tube) and centrifuged at 12,000 rpm for 30s. Discarded flow-through and placed the spin column into the collection tube.

- 500 µl Buffer GD was added to TIANamp Spin Column CB3, and centrifuged at 12,000 rpm for 30s, then discarded the flow-through and placed the spin column into the collection tube.

- 700 µl Buffer PW was added to TIANamp Spin Column CB3, and centrifuged at 12,000 rpm for 30s. The flow-through was discarded and placed the spin column into the collection tube.

- 500 µl Buffer PW was added to TIANamp Spin Column CB3, and centrifuged at 12,000 rpm for 30s. The flow-through was discarded and placed the spin column into the collection tube.

- Centrifuged at 12,000 rpm for 2 min to dry the membrane completely.

The resident ethanol of buffer PW may have some affection in downstream application.

- The TIANamp Spin Column CB3 was placed in a new clean 1.5 ml microcentrifuge tube and pipette 50-200 µl Buffer TE or distilled water directly to the center of the membrane. Incubated at room temperature (15–25°C) for 2-5 min, and then centrifuged for 2 min at 12,000 rpm.

Table 2.2 Apparatus details used for molecular identification of the isolates.

Name of the apparatus	Model	Company	Country of origin
Centrifuge	WiseSpin CF-10	Wisd Laboratory Instruments	Germany
Gel electrophoresis	Mini-Sub Cell GT Cell	Bio-Rad Laboratories, Inc.	USA
Thermal Cycler	GeneAtlas 482/485	Astec	Japan
Gel documentation	AlfaImager MINI	ProteinSimple	California, USA
Spectrophotometer	NanoDrop 2000	Thermo Scientific	USA
Sequencer	Genetic Analyzers 3130	Applied Biosystems	USA

In the Polymerase Chain Reaction the DNA to be amplified was denatured by heating the sample in the presence of DNA polymerase and excess dNTPs, the oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete “short product” which accumulates exponentially with each successive round of amplification. This can lead to the many million fold amplification of the discrete fragment over the course of 20 to 30 cycles.

The 16S rRNA gene of the isolated bacteria were amplified by a polymerase chain reaction (PCR) with the forward and reverse primers which correspond to nucleotides 8F–1492R, 8F–806R, 8F–907R, 515F–806R, 515F–907R and 515F-1492R 16S rRNA gene sequence, respectively (Table 2.3). The amplified PCR product was cloned and single forward strand was sequenced using the dideoxy-chain termination method. The 16S rRNA gene sequences were obtained from the GenBank, DDBJ, and EMBL database. Working steps followed in this study was presented in Figure 2.9 in brief.

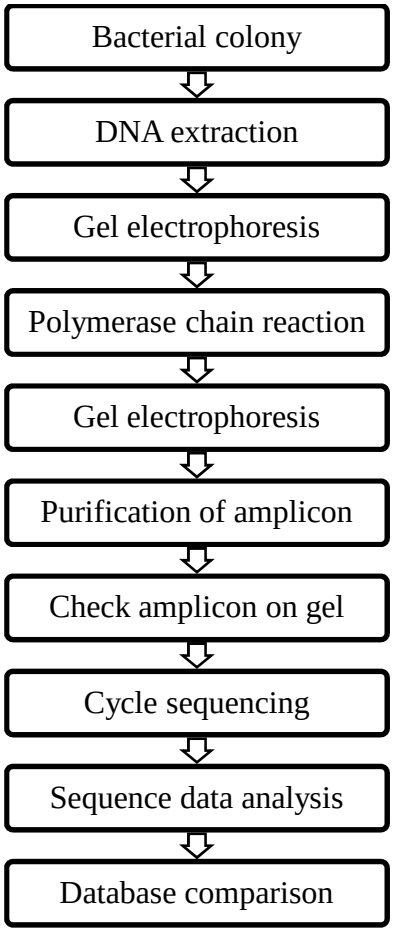


Figure 2.9 Steps of sequence identification.

Table 2.3 Primers and their sequence used in this study.

Primers*	**Sequence (5å-3å)**	**Target group**	**Reference**
8F	AGAGTTTGATCCTGGCTCAG	Universal	Turner *et al*. 1999
515F	GTGCCAGCMGCCGCGGTAA	Universal	Turner *et al*. 1999
806R	GGACTACCAGGGTATCTAAT	Universal	McBain *et al*. 2003
907R	CCGTCAATTCMTTTRAGTTT	Universal	Lane 1991
1492R	GGTTACCTTGTTACGACTT	Universal	Turner *et al*. 1999

Note: *Numbered primers are named for the approximate position on the *E. coli* 16S rRNA molecule.

These primers are frequently used as universal for genomic bacterial DNA. The PCRs used here were performed in a final volume of 50 µl containing 10 µl of DNA, 5 µl of 10× PCR buffer (50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris HCl), 2.0 µl of dNTPs (5

mM), 2.0 µl of a 5 mM solution of each primer, 0.5 µl of *Taq* DNA polymerase at 5 U/µl, and 28.5 µl of double-distilled sterile water. We used 1 cycle of denaturation at 95°C for 3:30 min, followed by 35 cycles of denaturation at 95°C for 45s, annealing at 49°C for 45s, and extension at 72°C for 1:30 min, and a final extension round at 72°C for 10 min. The PCR amplicons were separated electrophoretically in a 1% agarose gel and visualized after ethidium bromide staining.

Agarose gel electrophoresis: The amplified products were separated by electrophoresis of 10 µL (7 µL PCR product + 3 µL loading dye, Bromophenol blue) of the reaction product in 1.0% agarose gel (wv^{-1}) in Tris-Borate buffer (0.089M Tris, 0.089M boric acid, and 0.002M EDTA, pH 8), stained with ethidium bromide (1.6 mg/ml). The gel electrophoresis was carried out at 70 V at room temperature for ~ 1.0 hour in electrophoresis unit (Bio-Rad, USA) and DNA bands were visualized using UV transilluminator in gel documentation system. A 1 kb DNA ladder was used as molecular weight markers.

PCR product purification protocol: The PCR products were purified using TIANquick Midi purification kit (Tiangen, China) following the manufacturer's protocol.

- Column equilibration: 500 µl Buffer BL was added to the Spin Column CB2. Centrifuged for 1 min at 12,000 rpm (~13,400 × g) in a table-top microcentrifuge. The flow-throw was discarded, and then placed Spin Column CB2 in the collection tube.

- 5 volumes of Buffer PB was added to 1 volume of the PCR reaction or enzymatic reaction and mixed.

- The mixture was transferred to the Spin Column CB2 and stand for 2 min at room temperature (15-25°C). Centrifuged for 30~60s at 12,000 rpm (~13,400 × g) in a table-top microcentrifuge. The flow-throw was discarded, and then placed Spin Column CB2 back into the same collection tube.

- To wash, 600 µl Buffer PW was added to the Spin Column CB2 and centrifuged for 30-60s at 12,000 rpm (~13,400 × g). The flow-through was discarded, and placed Spin Column CB2 back in the same collection tube.

- The Spin Column CB2 was washed with 600 µl Buffer PW and centrifuged for 30-60s at 12,000 rpm (~13,400 × g). The flow-through was discarded, and centrifuged for an additional 2 min to remove residual wash buffer PW.

- The Spin Column CB2 was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 30-50 µl Buffer EB or deionized water (pH 7.0-8.5) was added to the center of membrane, let the column stand for 2 min, and centrifuged for 2 min at 12,000 rpm (~13,400 × g).

- Alternatively, for increased DNA concentration, the solution gained from step 6 was added to the center of membrane again, let the columns stand for 1 min, and then centrifuged.

2.4.19.2 Estimation of DNA

The total DNA yield and quality were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, USA).

2.4.19.3 16S rRNA gene sequencing and data analysis

In 1977, two different methods for sequencing DNA were developed, namely, the chain termination method and the chemical degradation method. Both methods were equally popular to begin with, but, the chain termination method soon become more popular and this method is more commonly used today. This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis. The fixed laser beam excites the fluorescently labeled DNA bands and the light emitted is detected by sensitive photodetectors. DNA sequence data is the most accurate and definitive way to identify microbes because the microbes may be identified by base pair to base pair of the nucleic acid. The DNA sequences of the variable regions form the basis of phylogenetic classification of microbes. By sequencing broad range PCR products, it is possible to detect DNA from almost any bacterial species. After comparing the resulting sequences with known sequences in GenBank or other databases, the identity of the unknown bacteria can be revealed. Since the 1990s, 16S rDNA sequencing has become a powerful tool, which is used more and more in microbial detection and identification algorithms, especially for unusual, non-culturable, fastidious and slow growing pathogens, or after antibiotics that have been administered to the patient. Such a technique as this is becoming a routine method of detection and identification of bacteria, thus overall helping to combat infectious diseases (Millar *et al*. 2007). Sanger sequencing workflow using dye terminator technology was followed for the present study (Appendix IV).

Sequencing analysis was performed on a ~800 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA sequences were aligned and compared with other 16Sr RNA genes in the GenBank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16S rRNA gene sequences were deposited to Genbank using BankIt submission tool.

2.4.19.4 Phylogenetic analysis

Phylogenetic analysis is the process used to determine the genetic evolutionary connections between species. The results of an analysis can be drawn in a hierarchical diagram called a cladogram or phylogram (phylogenetic tree). The branches in a tree are based on the hypothesized evolutionary relationships (phylogeny) between organisms. Each member in a branch, also known as a monophyletic group, was assumed to be a descendent from a common ancestor. Although originally, phylogenetic trees were created using morphological characteristics, the development of molecular biology has provide a molecular mean for determination the evolutionary relationships through matching patterns in nucleic acid and protein sequences during evolution, it is very common for a gene to be duplicated. The copies continue to evolve separately, resulting in at least two similar instances of the same gene along the genome of a species (Saitou and Nei 1987).

Phylogenetic analysis also gives the evidence how the pathogenic bacterium evolved. Modern phylogeny methods information extracted from mainly DNA and protein sequences after aligning several of these sequences and using only blocks which were conserved in all the examined species (Martínez-Murcia *et al*. 1992).

Procedure of nucleotide sequence analysis:

- Nucleotide sequence obtained from genetic analyzer.
- blastn search was performed through NCBI BLAST search engine.

- First 25 blast match were selected from the match except uncultured bacterium clone and complete sequence of any matched bacterial strain.

- Distance to tree result (this tree was produced using BLAST pair wise alignments).

  BLAST computes a pair wise alignment between a query and the database sequences searched. It does not explicitly compute an alignment between the different database sequences (i.e., does not perform a multiple alignment). For purposes of this sequence tree presentation an implicit alignment between the database sequences is constructed, based upon the alignment of those (database) sequences to the query. It may often occur that two database sequences align to different parts of the query, so that they barely overlap each other or do not overlap at all. In that case it is not possible to calculate a distance between these two sequences and only the higher scoring sequence was included in the tree.

- Neighbor joining tree method was used.

- Maximum sequence difference was 0.75.

- Sequence level was taxonomic name (sequence ID).

- Collapse mode was custom and distance showed.

- Unrooted tree was downloaded as Newick format.

- The guide tree was downloaded into a text file in Newick or Nexus format (recognized by popular phylogenetic analysis software). Node labels can be changed with the Sequence Label option (below, to the right). Internal nodes whose all children have the same blast name and subtrees collapsed by a user are labeled with Blast Name.

  Newick and Nexus formats do not support collapsed subtrees, therefore fully expanded tree was always downloaded.

- The Newick format tree was edited with MEGA version 6.0 software.

CHAPTER 3: RESULTS AND OBSERVATIONS

3.1 Observation on the spot

Pond I (Kakanhat, Rajshahi): Semi-intensive mixed carp culture system was followed by the producer. Fish fry were collected from renowned hatchery and there were no signs of any kind of abnormalities or illness before they first got the dead fish on floating condition. Surrounding environment, dike, water colour of the pond was good (Table 3.1) as well as there was no complain about growth rate. They applied NaCl and lime in the affected pond at the fourth day and transferred fresh fishes (fishes without any symptoms of disease) into another pond where water was stocked from deep tube well. Then the owner harvested all the fishes and sold out at a lower price. On the spot water temperature, pH and salinity were recorded as 19.5°C, 7.9 and 0 ppt respectively at 8:15 am (December 2012).

Pond II (Darusha, Rajshahi): Mixed carp culture system was practiced in pond II where *Labeo* sp. and *Catla* were the main species along with other exotic carps. The physiography of the pond was not so good because it was somewhat like a depression and fully surrounded by paddy field and water came out from those field in case of heavy rainfall or over flow of irrigation (Table 3.1). So the chemical properties of water was regulated by the fertilizer and/or pesticide applied to the field and there is a chance to affect the stocked fish population. Water temperature, pH and salinity were recorded on the spot as 26.7°C, 8.2 and 1 ppt respectively (July 2013).

Pond III (Ullapara, Sirajganj): Semi-intensive carp (*Catla catla*) monoculture was practiced by the grower, the pond resides at locality and heavily used for homestead use (Table 3.1). Because of the size and water depth of the pond it was quite difficult to manage the parameters for ideal fish culture. Bottom soil was heavily black clayish with bad odour and gas and may be this was the main reason for epidemic fish mortality. The producer applied NaCl and lime in huge quantity and use antibiotic and other available medicine as prescribed by the company representatives without any investigation. Water temperature, pH and salinity were recorded as 17-18°C, 7.8-8.2 and 2-3 ppt respectively (August 2013). The pond owner of the third pond faced a huge economic loss because all the stocked fishes were died carrying same symptoms (mainly pale gills).

Table 3.1 Physiographical characteristics of the ponds (N=3).

Parameters	Pond I (Kakanhat)	Pond II (Darusha)	Pond III (Ullapara)
Pond type	Stocking	Stocking	Stocking
Structure	Rectangular	Square	Irregular
Area (acres) approx.	3.4	2.31	8.3
Bottom soil	Typical	Typical	Dark clay with bad odour
Dike	Well, used for vegetable gardening	Not so good but no trees around	With large trees at each dimension
Water source	Deep tube well	Rainwater	Natural/ground water
Water depth (meters)	2.75	1.2	4.8
Water colour	Greenish	Brownish	Greenish
Productivity	Medium	Below the limit	Below the limit
Homestead use	No	Occasionally	Each and every purpose

3.2 Investigation for the identification of the bacteria

Part I: Gross clinical signs of disease

Part II: Internal abnormalities relevant to bacterial fish pathogens

Part III: Identification of bacterial isolates

Part I: Gross clinical signs of disease

On the spot after collection screening of diseased fishes have been done on the basis of behavioural and physical abnormalities (sluggish and erratic movement, haemorrhage at the base of fins and on the fins, fin damage, pale gill, white spots on the head, haemorrhage around the vent, haemorrhage on the body surface, scale erosion).

Part II: Internal abnormalities relevant to bacterial fish pathogens: Apparent during post-mortem examination

No abnormalities were found in liver, kidney or other internal body parts. May be there were the first stage of infection on the body surface.

Part III: Identification of bacterial isolates

The most common shortcomings in diagnosis of fish diseases concern the identification of bacterial isolates. There are three schools of thought, namely those that rely on serology, molecular techniques, and those relying on more conventional phenotypic tests.

Labeo sp., *C. catla*, *Cirrhina* sp., *P. sarana* with haemorrhagic lesions at caudal peduncle, base of pelvic and anal fin, scale erosion, reddened caudal fin were sampled from Kakanhat area at the end of the year 2012 (Plate 3.1). Last week of July 2013 information was from the Darusha area where one *L. rohita* was found at moribund condition with haemorrhagic skin at many areas of the body. At last, on 30 August 2013 fry of *C. catla* with pale gill and white spot on head were collected at inverted floating moribund condition from the village of Purnimagati union under Ullapara upazilla of Sirajganj district.

Primarily after 24 hours of incubation from the mother culture plates, different sized and coloured colonies were separately sub-cultured subsequently on to nutrient agar plates to obtain fresh isolated colony (Plate 3.2, 3.3). After a period of time when the colonies were found as pure, isolated with same colony characteristics they were given symbol and stored for further experiment (Table 3.2).

Table 3.2 Isolate's name used in the present study and characters on which they were primarily differentiated for sub-culture.

Isolate symbol	Stands for	Sample from	Fish species	Colony colour	Colony size
CK	Creamy Kakanhat	Mucilage	*Puntius, Labeo, Cirrhinus*	Cream	Large
WK	White Kakanhat	Skin lesion	*Puntius, Labeo, Cirrhinus*	White	Large
YK	Yellow Kakanhat	Skin lesion	*Puntius, Labeo, Cirrhinus*	Pale yellow	Medium
CD	Creamy Darusha	Skin lesion, Gill	*Labeo*	Cream	Large
YD	Yellow Darusha	Skin lesion, Gill	*Labeo*	Orange	Large
CYD	Creamy-Yellow Darusha	Skin lesion, Gill	*Labeo*	Cream	Large
SW/Cr	Water Sample Creamy	Pond water	*Catla*	White	Large
SW/Yel	Water Sample Yellow	Pond water	*Catla*	Orange	Large
GSH/Cr	Gill Skin Head Creamy	Gill, Skin lesion	*Catla*	Cream	Large

Puntius sp. with haemorrhagic lesion

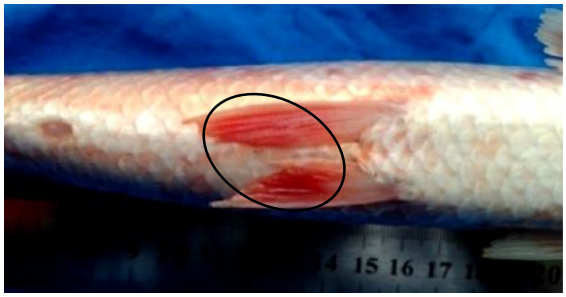
Puntius sp. with haemorrhagic lesion

Labeo sp. with scale erosion

L. rohita with haemorrhagic skin

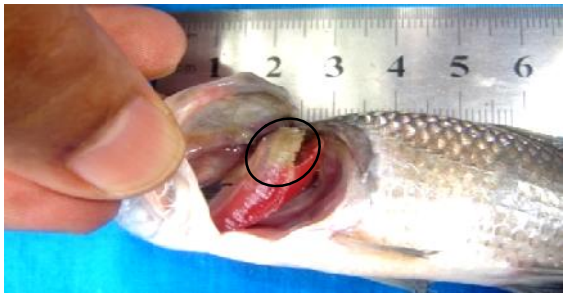
L. rohita with haemorrhagic skin

L. rohita with haemorrhagic caudal fin

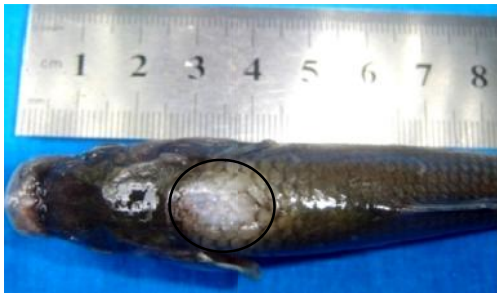


L. rohita with haemorrhagic pelvic fin

L. rohita with haemorrhagic caudal fin



C. catla with affected gills



C. catla with dermal lesion

Plate 3.1 Collected diseased fishes with gross clinical signs of diseases.

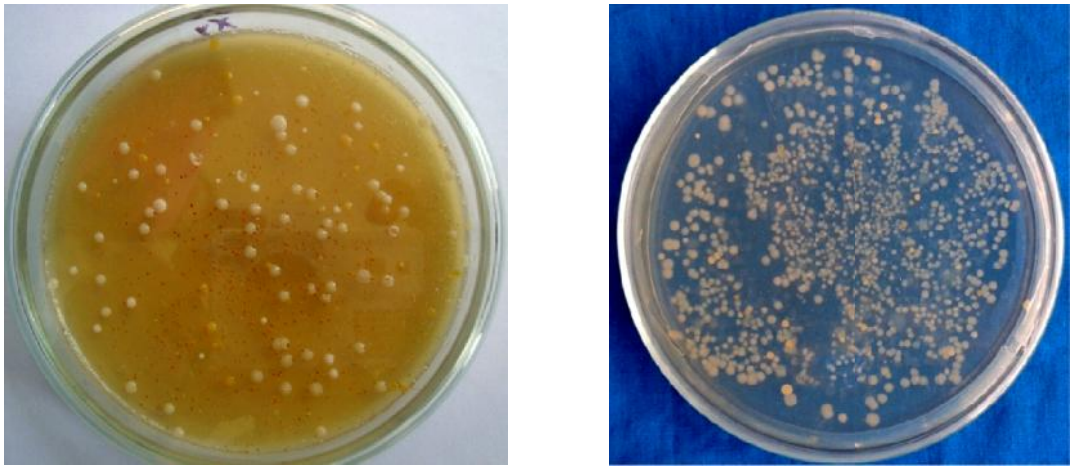


Plate 3.2 Primary or mother culture plates bearing different sized and coloured colonies.

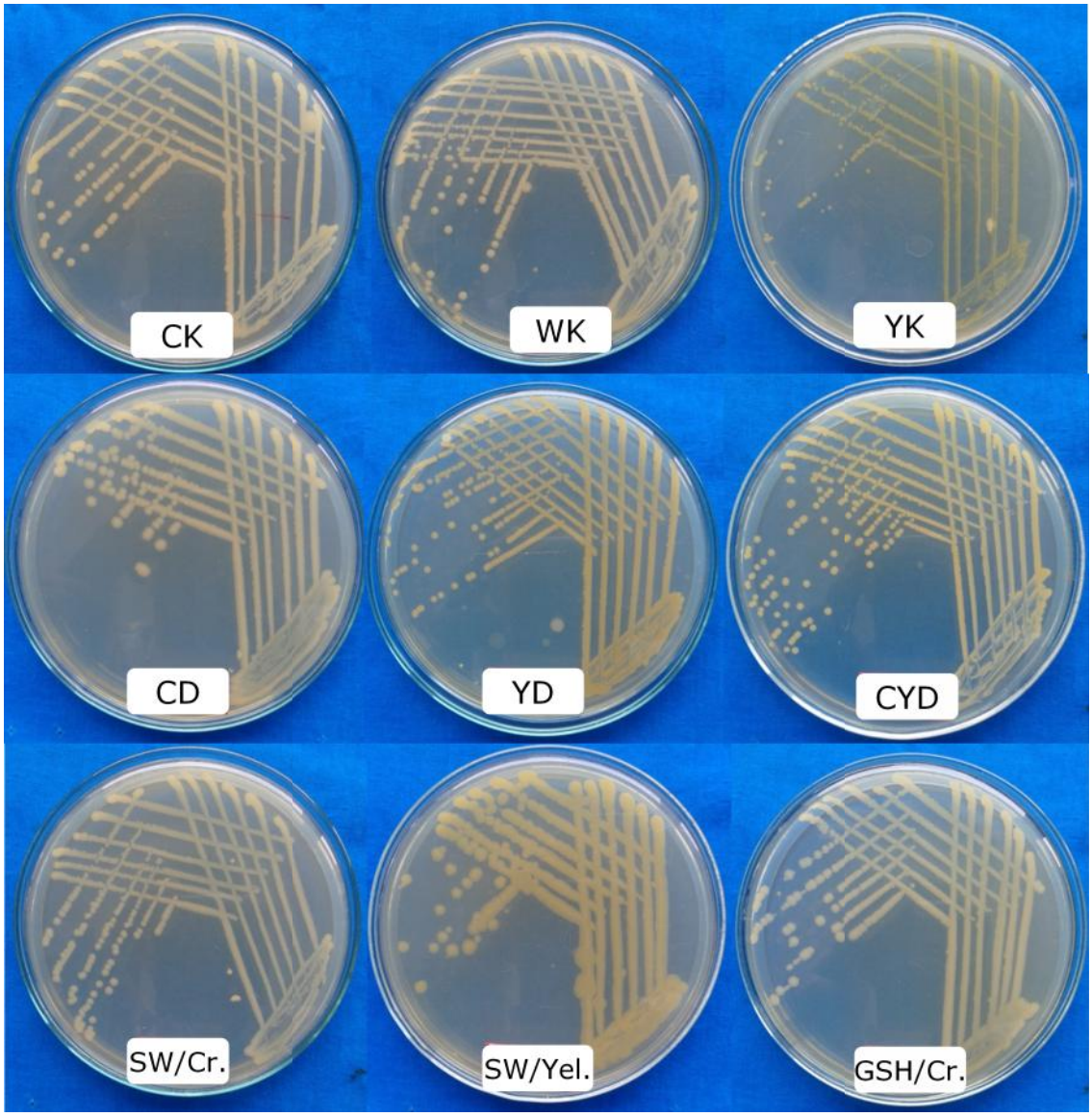


Plate 3.3 Culture plates bearing isolated colonies of the isolates (N=9).

3.2.1 Colony morphology and pigmentation

The initial interpretation of bacterial growth on primary culture media, which usually follows the first 24 to 48 h of incubation, is an opportunity for the skilled microbiologist to make a preliminary identification and to decide what additional tests and procedures must be performed to arrive at a definitive identification (Isenberg 2007).

Hence, colony morphology and pigmentation was recorded from young colonies, i.e. shortly after growth on the basis of continuous observation and the results are presented in Table 3.3. Except density the three isolates CK, WK and SW/Cr resembles the same colony characteristics. Two isolates CD and GSH/Cr gave the same characteristics on incubation of 24 hours at 37°C. Isolates YD and CYD bearing the same colony morphology where YD was more contrast in colour than that of CYD. Isolates YK and SW/Yel were different in colony colour and morphology.

Table 3.3 Terms to describe gross colonial morphology (according to Clinical Microbiology Procedures Handbook, American Society for Microbiology).

Bacterial isolates	Size	Shape/ Form	Elevation	Margin	Colour	Surface appearance	Density	Consistency
CK	Large	Circular	Convex	Entire	Cream	Glistening, Smooth	Opaque	Butyrous
WK	Large	Circular	Convex	Entire	White	Glistening, Smooth	Opaque	Butyrous
YK	Medium	Circular	Convex	Entire	Pale yellow	Glistening, Smooth	Transparent	Viscid
CD	Large	Circular	Umbonate	Undulate	Cream	Granular	Opaque	Butyrous
YD	Large	Circular	Flat	Entire	Orange	Dull, Smooth	Opaque	Butyrous
CYD	Large	Circular	Flat	Entire	Cream/ Orange	Dull, Smooth	Opaque	Butyrous
SW/Cr	Large	Circular	Convex	Entire	White	Glistening, Smooth	Translucent	Butyrous
SW/Yel	Large	Irregular	Raised	Undulate	Orange	Rough	Opaque	Brittle/ Friable
GSH/Cr	Large	Circular	Umbonate	Undulate	Cream	Granular	Opaque	Butyrous

Note: **Size:** Large = greater than 1 mm in diameter, Medium = 1 mm in diameter, Small = less than 1 mm in diameter
Shape/Form: Circular, Filamentous, Irregular, Punctiform, Rhizoid, Spindle
Elevation: Flat, Raised, Convex, Dome shaped, Umbonate, Umbilicate
Margin (edge of colony): Entire, Undulate, Lobate, Erose, Filamentous, Curled
Colour: White, Black, Cream, Orange etc.
Surface appearance: Glistening, Smooth, Granular, Dull, Rough, Creamy
Density (ability to see through colony): Opaque = cannot see through the colony, Transparent = can see through the colony, Translucent = only with light shining through
Consistency (best observed by picking up a colony with a loop or needle): Butyrous (buttery), Viscid (sticky), Friable (crumbles easily), Brittle, Membranous

3.2.2 The Gram-staining

Six isolates were found as Gram-negative while other three were Gram-positive according to both the tests performed. This reaction was performed with smears from young cultures and the results are presented in Table 3.4 and Plate 3.4.

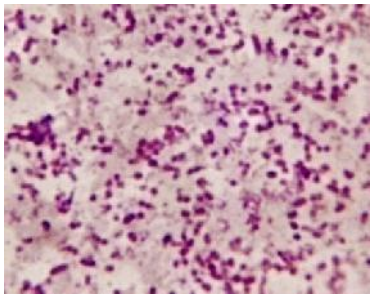
Table 3.4 Gram characteristics and shape of the isolates (N=9).

Isolates	Gram-reaction	KOH stringing test	Shape of bacteria
CK	- ve	+ ve	Short rod
WK	- ve	+ ve	Short rod
YK	- ve	+ ve	Rod
CD	- ve	+ ve	Straight rod
YD	+ ve/- ve	- ve	Ovoid to short rod
CYD	+ ve	- ve	Ovoid to short rod
SW/Cr	- ve	+ ve	Short rod
SW/Yel	+ ve	- ve	Large rod
GSH/Cr	- ve	+ ve	Straight rod

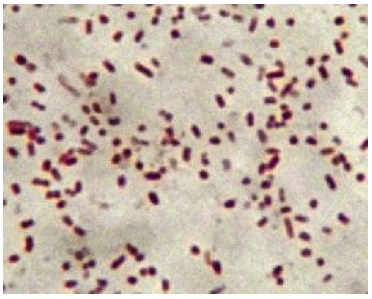
There was an isolate named as YD collected from *Labeo* sp. from Darusha area with haemorrhagic lesion showed variable results with Gram reaction. All the isolates were rod shaped while isolates CK, WK and SW/Cr showed same results and they were short rod, CD and GSH/Cr isolates were found straight rod. YD and CYD isolates were short rod with ovoid shape and isolate SW/Yel was large in shape (Table 3.4, Plate 3.4). Gram characters of the isolates were confirmed by alternative test with KOH string test. Six isolates named CK, WK, YK, CD, SW/Cr and GSH/Cr became viscid within 30 seconds of mixing with 3% KOH solution where YD, CYD and SW/Yel isolates were incapable to change the viscosity of the KOH solution (Table 3.4).

3.2.3 Biochemical identification

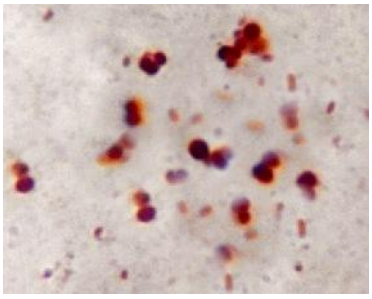
Biochemical identification of a bacterium proceeds through a number of steps. A pure subculture of the organism is used to perform primary identification tests and to inoculate biochemical identification sets (secondary identification tests), composed of either in-house prepared media, or commercial identification sets such as API. The identification sets are incubated at the appropriate temperature and for the appropriate time for reactions to occur. The media and reagents used in the biochemical identification along with information on growth characteristics and reagent reactions are presented here.



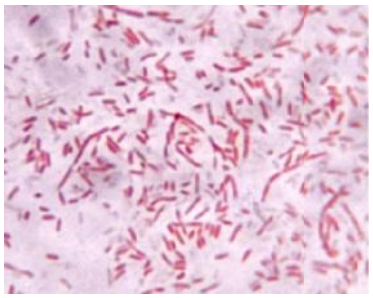
CK Gram -ve



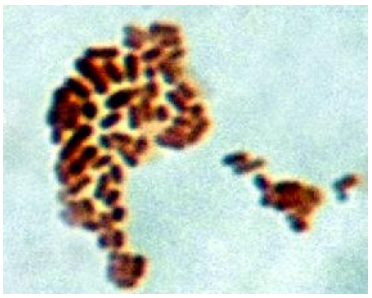
WK Gram -ve



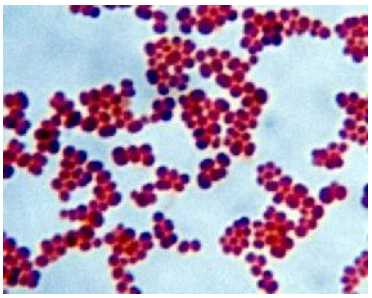
YK Gram -ve



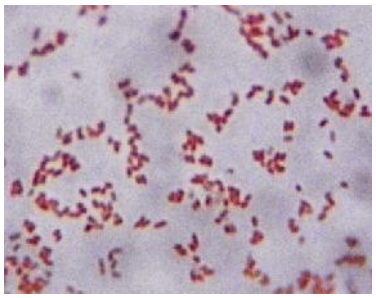
CD Gram -ve



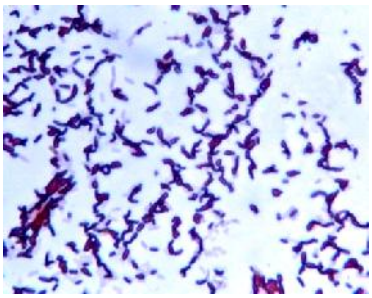
YD Gram +ve



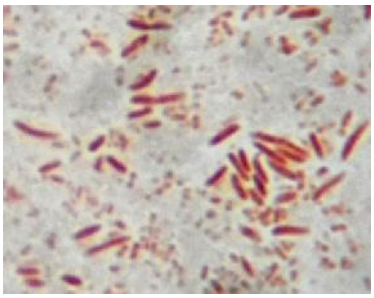
CYD Gram +ve



SW/Cr Gram -ve



SW/Yel Gram +ve



GSH/Cr Gram -ve

Plate 3.4 Gram characters and shape of the bacterial isolates, 16×100X (N=9).

Primary tests: The primary tests include microscopic examination of smears, in particular the Gram stain, catalase, oxidase, motility, and growth on MacConkey agar (MCA). Information on the performance of these tests and their interpretation is described and methods of media and reagent preparation are described in materials and methods.

Secondary tests (biochemical identification sets): Biochemical identification is achieved by secondary identification tests, which are the main tests used to identify an organism to species level.

3.2.3.1 Lactose fermentation test (growth on MacConkey agar, MCA)

Among the nine isolates six showed their growth on this media while two of them are lactose fermenter. Gram-positive bacteria cannot grow on to this medium because of the presence of bile salt and crystal violet. From the result we confirmed that six isolates (CK, WK, YK, CD, SW/Cr and GSH/Cr) are Gram-negative while other three (YD, CYD and SW/Yel) are Gram-positive. Among the six positive isolates only two (CK and WK) can ferment lactose while other four cannot ferment lactose. Growth characteristics and results were observed and recorded after inoculation of the young isolated single colony on previously made plates which were incubated for overnight at ~ 37°C and are presented in Table 3.5, Plate 3.5.

3.2.3.2 Citrate Utilization test (growth on Simmons citrate, SC agar)

Among the nine isolates six showed their growth on this media. Gram-positive bacteria cannot grow on to this medium. From the result we confirmed that six isolates are Gram-negative while other three are Gram-positive. Growth characteristics and results were observed and recorded after inoculation of the young isolated single colony on previously made slant tubes which were incubated for overnight at ~37°C and are presented in Table 3.6, Plate 3.6.

3.2.3.3 Catalase activity

Catalase is an enzyme produced by most aerobic organisms to detoxify H_2O_2. This may be H_2O_2 produced naturally in aerobic cells, or H_2O_2 produced by immune cells as a toxic weapon against pathogens. When cells containing catalase are mixed with H_2O_2, the peroxide is converted to H_2O and O_2, with vigorous bubbling. Few microbial organisms, such as *Streptococcus* and *Enterococcus*, are catalase-negative, so this test is useful for distinguishing these species from *Staphylocccus*. Cells often cease to produce catalase after an extended culture period, so this test was done within 24 hrs. Isolates CK, WK, SW/Cr and SW/Yel were found as catalase positive while YD and CYD were weak positive on catalase activity. YK, CD and GSH/Cr did not produce bubbles when given to H_2O_2 as they are catalase negative (Table 3.7, Plate 3.7).

Table 3.5 Growth characteristics of the isolates (N=9) on MacConkey agar (MCA) media.

Isolates	Growth and colour	Gram characters	Lactose Fermentation	Remarks
CK	Growth, Pinkish red	-ve	Lactose fermenters	Growth occurred and all colonies are noticeably pinkish red. Acid has been produced.
WK	Growth, Pinkish red	-ve	Lactose fermenters	Growth occurred and all colonies are noticeably pinkish red. Acid has been produced.
YK	Growth, Yellowish-Creamy	-ve	Lactose non-fermenters	Growth occurred and colonies are colorless. No acid has been produced.
CD	Growth, Yellowish-Creamy	-ve	Lactose non-fermenters	Growth occurred and colonies are colorless. No acid has been produced.
YD	No growth occurred	+ve	No growth	Bile salt and crystal violet in the medium inhibited the growth of the organism.
CYD	No growth occurred	+ve	No growth	Bile salt and crystal violet in the medium inhibited the growth of the organism.
SW/Cr	Growth, Yellowish-Creamy	-ve	Lactose non-fermenters	Growth occurred and colonies are colorless. No acid has been produced.
SW/Yel	No growth occurred	+ve	No growth	Bile salt and crystal violet in the medium inhibited the growth of the organism.
GSH/Cr	Growth, Yellowish-Creamy	-ve	Lactose non-fermenters	Growth occurred and colonies are colorless. No acid has been produced.

3.2.3.4 Oxidative-fermentative (O-F) test

The pathways used to metabolize a sugar under aerobic conditions differ from those used under anaerobic conditions. When oxygen is present, the process is called oxidation, and when oxygen is absent it is called fermentation. This test provides insight into the ability of the bacterium to use glucose by both methods. All the isolates showed same results for oxidative-fermentative test and found as fermentative type of bacteria (Table 3.7).

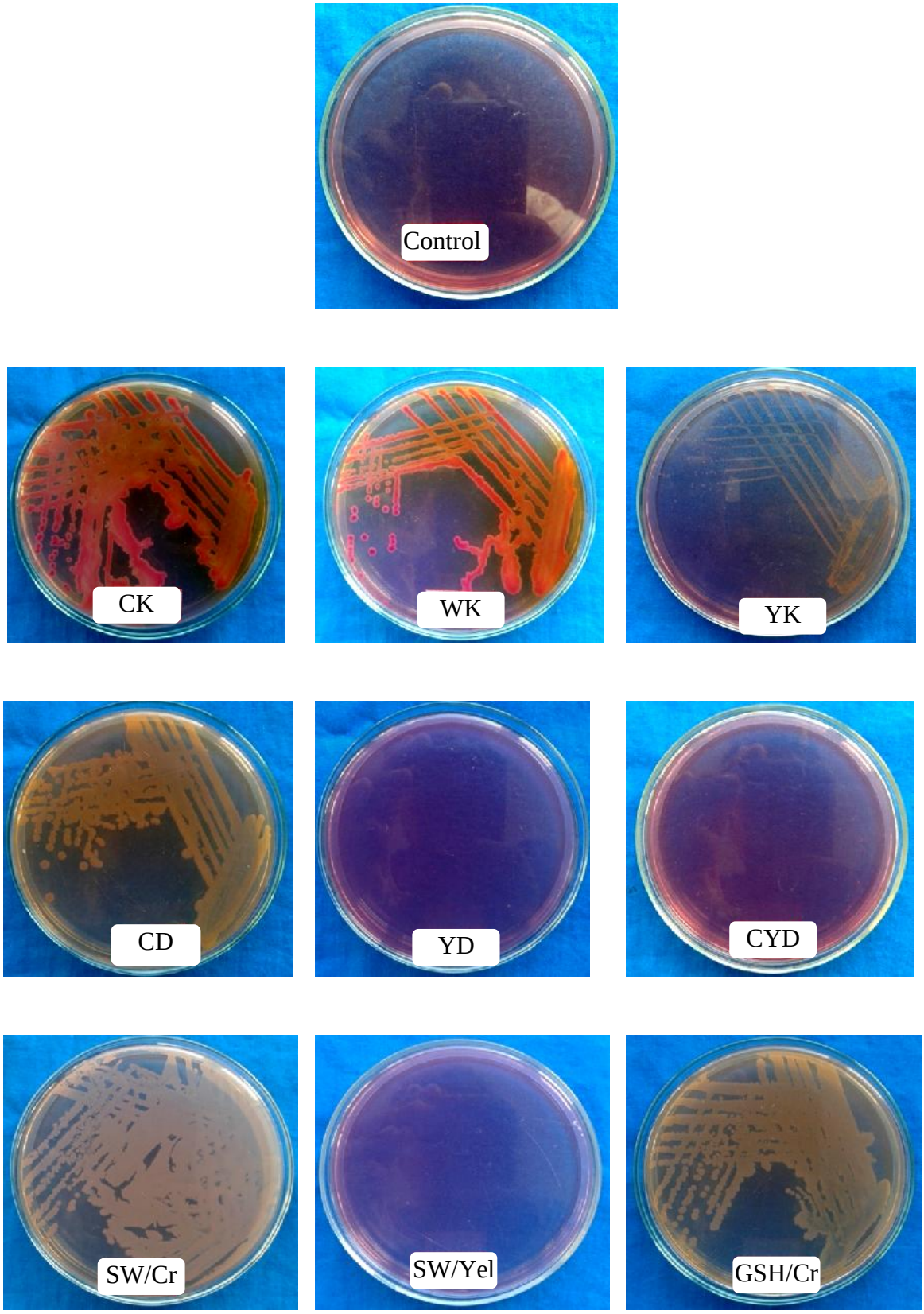


Plate 3.5 Growth of bacterial isolates (N=9) on MacConkey agar (MCA) media.

Table 3.6 Growth characteristics of the isolates (N=9) on Simmons Citrate (SC) agar.

Isolates	Growth	Gram characteristics and type	Citrate utilization	Remarks
CK	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition
WK	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition
YK	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition
CD	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition
YD	no change	+ve	---	---
CYD	no change	+ve	---	---
SW/Cr	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition
SW/Yel	no change	+ve	---	---
GSH/Cr	+ ve	- ve, enteric bacteria	Utilize citrate	turned from green colored acidic condition to royal-blue colored alkaline condition

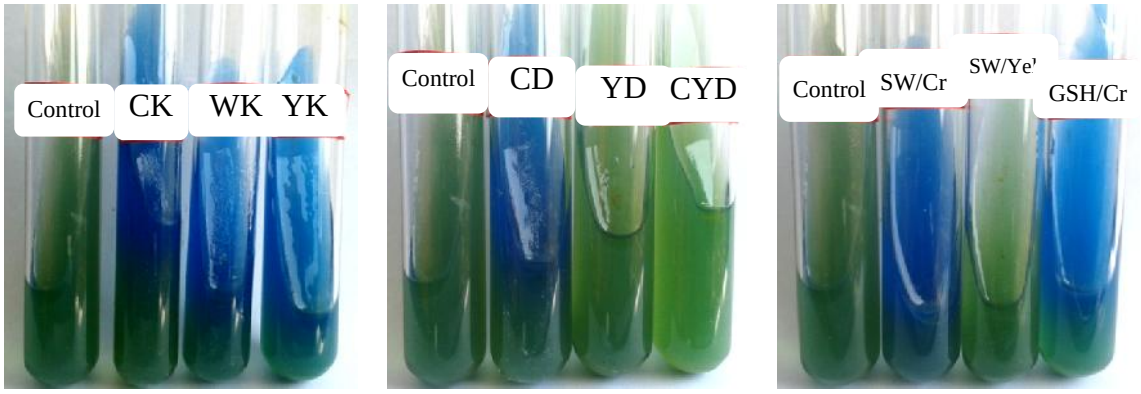


Plate 3.6 Bacterial isolates (N=9) along with control tube showing growth results in Simmons Citrate (SC) agar media.

3.2.3.5 Cytochrome-oxidase test

Three isolates (CK, WK and SW/Cr) finally found as different strains of *Klebsiella oxytoca* through 16S rRNA gene sequencing and showed the same results as they all were oxidase negative (Table 3.7, Plate 3.8). Isolates CD and GSH/Cr which revealed as *Aeromonas* sp. found as oxidase positive. *Brevibacillus* spp. (isolates YD and CYD) were found as weak oxidase positive. Sometimes they showed oxidase negative results. The genus is also catalase positive or negative. That is why known as variable for catalase activity, oxidase and Gram variable. Isolate YK (*Pantoea* sp.) and SW/Yel. (*Bacillus* sp.) are oxidase negative.

Table 3.7 Catalase production, oxidative-fermentative (O-F) and oxidase test results of the isolates (N=9).

Isolates	Catalase activity	Oxidative-fermentative	Oxidase
CK	+ ve	Fermentative	- ve
WK	+ ve	Fermentative	- ve
YK	- ve	Fermentative	- ve
CD	- ve	Fermentative	+ ve
YD	+ ve, weak	Fermentative	- ve
CYD	+ ve, weak	Fermentative	+ ve
SW/Cr	+ ve	Fermentative	- ve
SW/Yel	+ ve	Fermentative	- ve
GSH/Cr	- ve	Fermentative	+ ve

3.2.3.6 Growth on Triple Sugar Iron (TSI) agar

Fermentation of glucose and lactose and/or sucrose, production of gas, and acidic fermentation exhibits by the three isolates CK, WK and SW/Cr. Isolate YK fermented lactose and/or sucrose, but didn't grow in the anaerobic area. Only glucose was fermented but turned to the amino acids, production of gas, but no sulfur reduction, and peptone utilized by the two isolates CD and GSH/Cr. Isolates YD and CYD fermented sugars but didn't grow in the anaerobic area. Sugars fermented but cannot grow in the anaerobic area, neither sugars nor peptone catabolized by the isolate SW/Yel. Results are presented with detailed interpretation of the observation in Table 3.8, Plate 3.9.

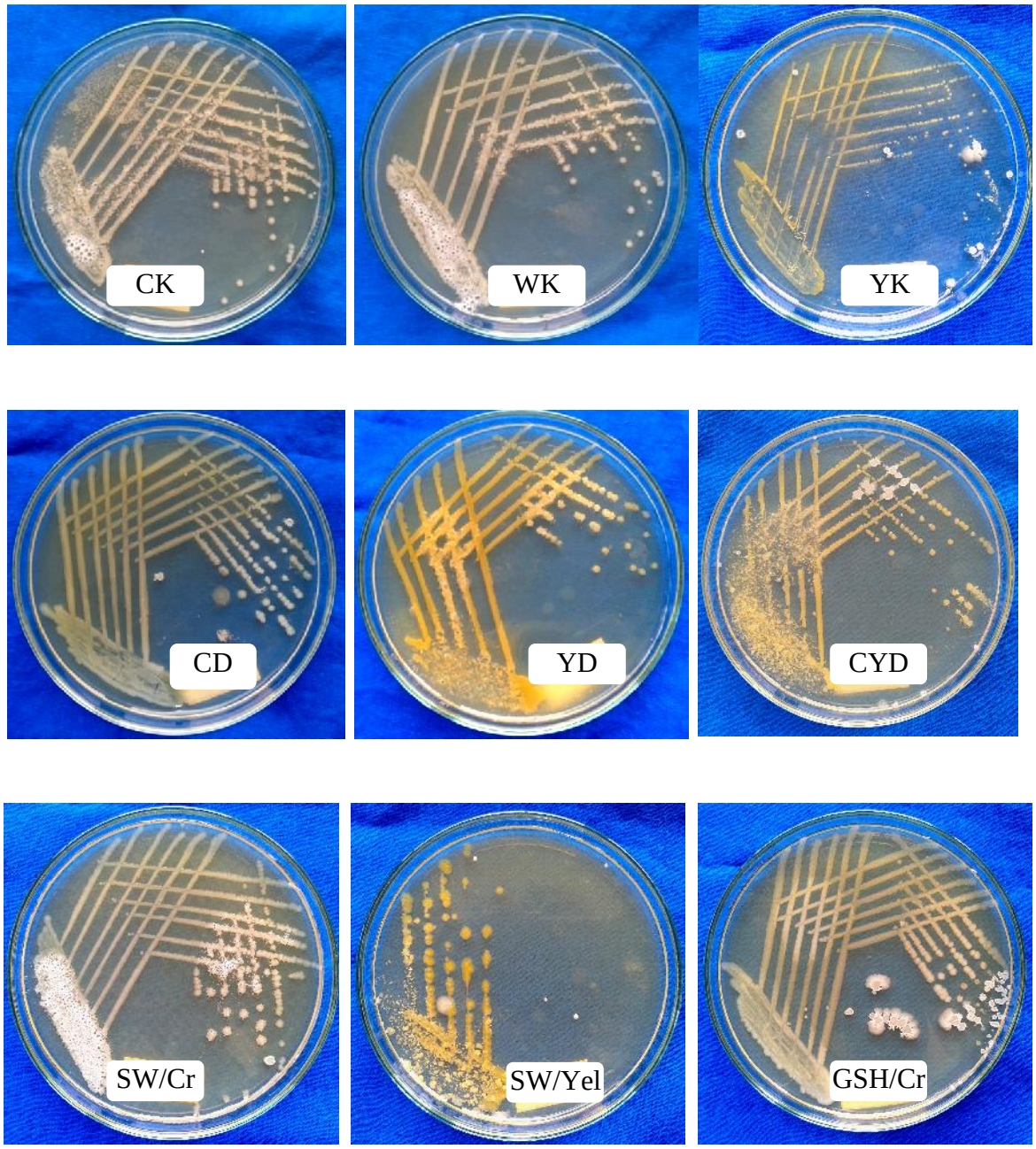


Plate 3.7 Catalase activity of the isolates (N=9).

Oxidase + ve Oxidase - ve

Plate 3.8 Results for oxidase test.

Table 3.8 Growth characteristics of the isolates (N=9) on Triple Sugar Iron (TSI) agar.

Isolates	Result (slant/butt)	Symbol	Interpretation of results
CK	Yellow/Yellow	A/A, Gas produced	Glucose and lactose and/or sucrose fermentation, gas produced, exhibits acidic fermentation
WK	Yellow/Yellow	A/A, Gas produced	Glucose and lactose and/or sucrose fermentation, gas produced, exhibits acidic fermentation
YK	Yellow/Yellow	A/A	Lactose and/or sucrose fermentation, ferment sugars but didn't grow in the anaerobic area of the butt
CD	Red/Yellow	K/A, Gas produced	Glucose fermentation only, gas produced, fermented the sugars but turned to the amino acids, but no sulfur reduction, peptone utilized
YD	Yellow/Red	A/NC	Fermented sugars but didn't grow in the anaerobic area of the butt
CYD	Yellow/ Red	A/NC	Fermented sugars but didn't grow in the anaerobic area of the butt
SW/Cr	Yellow/Yellow	A/A, Gas produced	Glucose and lactose and/or sucrose fermentation, gas produced, exhibits acidic fermentation
SW/Yel	Yellow/Red	A/NC	Ferment sugars but didn't grow in the anaerobic area of the butt, neither sugars nor peptone catabolized
GSH/Cr	Red/Yellow	K/A, Gas produced	Glucose fermentation only, gas produced, fermented the sugars but turned to the amino acids, but no sulfur reduction, peptone utilized

Note: A=acid production, K=alkaline reaction, NC= no change

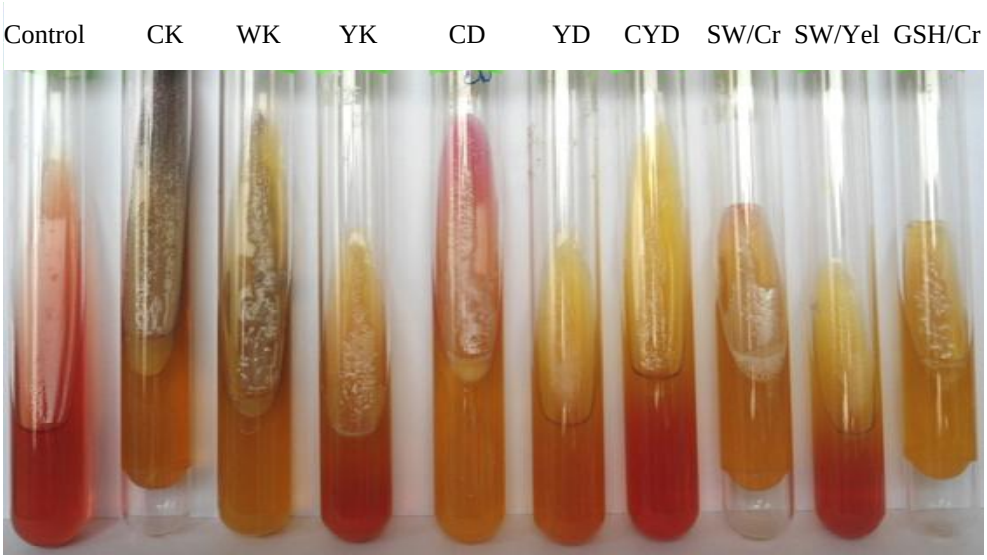


Plate 3.9 Growth results on Triple Sugar Iron (TSI) agar (N=9).

3.2.3.7 Growth on Sulfur Indole Motility (SIM) media

Production of hydrogen sulphide (sulphur reduction): None of the isolates reduces sulphur from the SIM media and no blackened seen up to third day of inoculation. This was also confirmed by TSI agar medium where no adverse results were found in relation to H_2S production (Table 3.8, 3.9, Plate 3.9, 3.10).

Indole production: Among the nine isolates examined here five (CK, WK, CD, SW/Cr and GSH/Cr) produced indole ring at the top of SIM media when added with Kovac's indole reagent after incubation of 24 hours at ~37°C. Moreover, the five isolates did not showed same intensity or manner while producing indole, some were strong and some were weak positive with Kovac's reagent (Table 3.9, Plate 3.10).

Motility: This was performed by SIM media, MIU media and observation on hanging drop method under microscope. Isolates CK and WK were non-motile through MIU media but SW/Cr showed motility on this though the three isolates finally identified as different strains of *Klebsiella oxytoca*. Isolates CK, WK and CD were observed and found as motile by means of polar flagella under microscope by hanging drop method. Motility was recorded for the isolates CD and GSH/Cr in SIM media and MIU media while YK, YD, CYD and SW/Yel were non-motile in both the media used (Table 3.9, Plate 3.10).

3.2.3.8 Urease (urea hydrolysis) and Motility Indole Urea (MIU) test

The isolates were tested via two different protocols to know their urease activity, one was through urea hydrolysis test and the other was growth on motility indole urea agar. Two tests revealed same results on the capacity to hydrolyze urea (Table 3.9, Plate 3.11, 3.12). Isolates CK, WK and SW/Cr were positive i.e. they can hydrolyze urea. CK and WK can hydrolyze urea within 22 hours while WK was the fastest and SW/Cr was slow in this process and take 34 hours to hydrolyze urea on the slant. In between isolates YD and CYD, identified as *Brevibacillus* sp. YD can hydrolyze urea where CYD cannot change the state. Isolates CD and GSH/Cr gave the same as they were unable to hydrolyze urea. Isolate SW/Yel was incapable to hydrolyze urea while isolate YK showed variable results.

Table 3.9 Growth characteristics of bacterial isolates (N=9) on SIM (sulphur reduction, indole production, motility test) and MIU (motility, indole, urea) media.

Isolates	Sulphur reduction	Indole production		Motility	
		SIM media	MIU media	SIM media	MIU media
CK	- ve	+ ve	+ ve	+ ve	-ve
WK	- ve	+ ve	+ ve	+ ve	-ve
YK	- ve	- ve	- ve	- ve	- ve
CD	- ve	+ ve	+ ve	+ ve	+ ve
YD	- ve	- ve	- ve	+ ve	- ve
CYD	- ve	- ve	- ve	+ ve	- ve
SW/Cr	- ve	+ ve	+ ve	+ ve	+ ve
SW/Yel	- ve	- ve	- ve	- ve	- ve
GSH/Cr	- ve	+ ve	+ ve	+ ve	+ ve

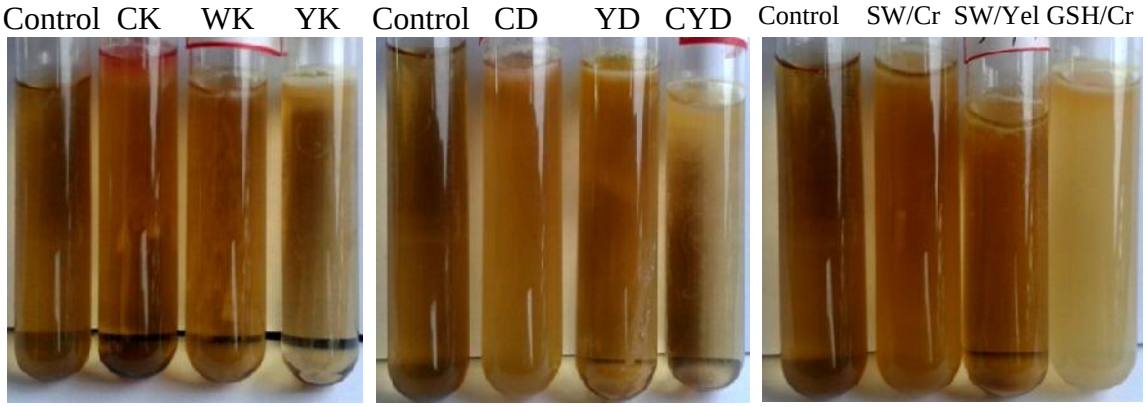


Plate 3.10 Bacterial isolates (N=9) along with control tube showing growth results in Sulphur Indole Motility (SIM) media.

3.2.3.9 Methyl red test and Voges-Proskauer reaction

Methyl red (MR) test and Voges-Proskauer (VP) reaction for the isolates were performed for the differentiation of the bacteria. Six isolates showed positive results for methyl red test while four of them were VP positive. In both the cases isolate YK and SW/Cr were showed positive results while only isolate YD was negative on both tests. Otherwise the rest of the isolates showed vice-versa results (Table 3.10, Plate 3.13, 3.14).

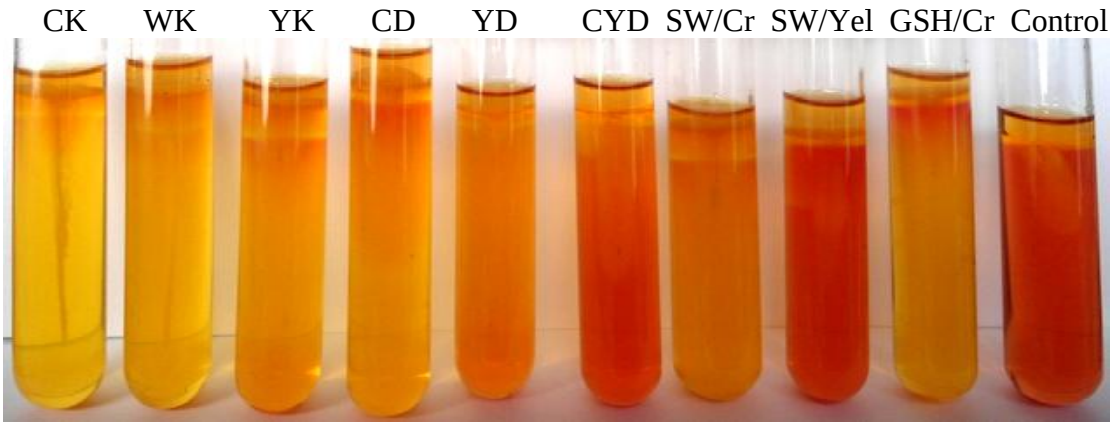


Plate 3.11 Response of the isolates (N=9) to the Motility Indole Urea (MIU) media.

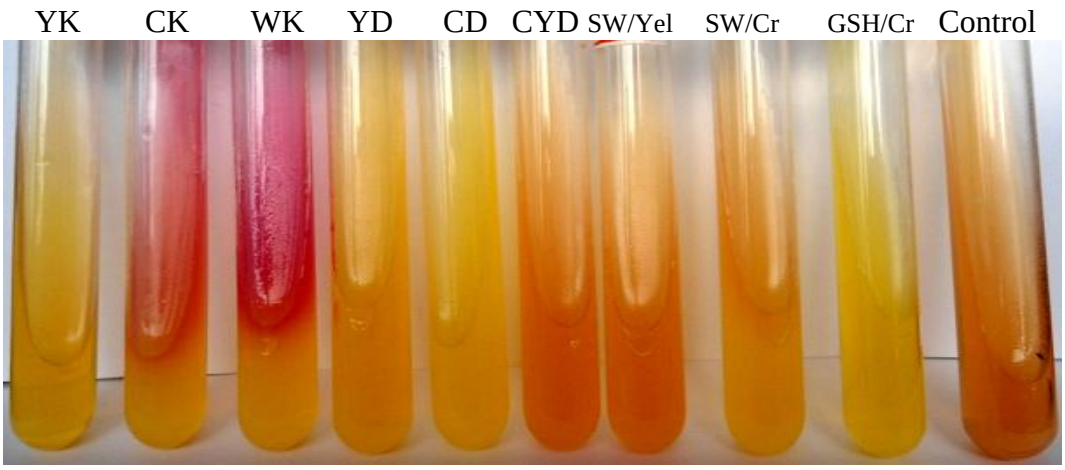


Plate 3.12 Growth results of the isolates (N=9) on urease (urea hydrolysis) test.

Table 3.10 Methyl red test and Voges-Proskauer reaction results for the isolates (N=9).

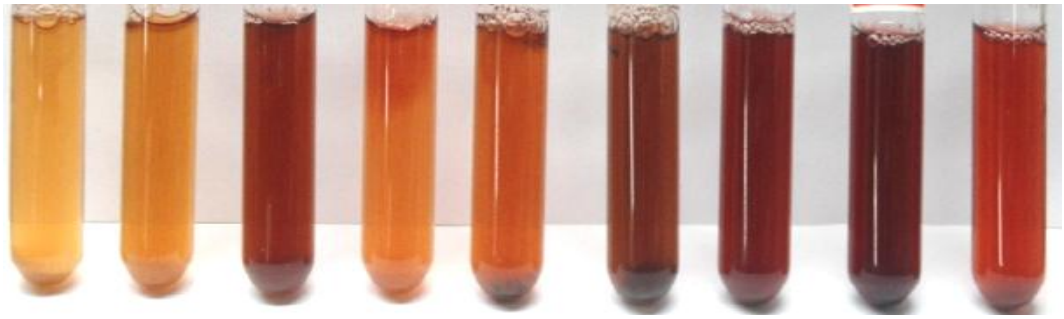
Isolates	Methyl red test	Voges-Proskauer reaction
CK	+ ve	- ve
WK	+ ve	- ve
YK	+ ve	+ ve
CD	+ ve	- ve
YD	- ve	- ve
CYD	- ve	+ ve
SW/Cr	+ ve	+ ve
SW/Yel	- ve	+ ve
GSH/Cr	+ ve	- ve

CK WK YK CD YD CYD SW/Cr SW/Yel GSH/Cr

+ve +ve +ve +ve -ve -ve +ve -ve +ve

Plate 3.13 Methyl red test of the isolates (N=9).

CK WK YK CD YD CYD SW/Cr SW/Yel GSH/Cr



-ve -ve +ve -ve -ve +ve +ve +ve -ve

Plate 3.14 Voges-Proskauer reaction of the isolates (N=9).

3.2.3.10 Acid production from sugars (carbohydrate/sugar utilization/fermentation test)

Sucrose, fructose, galactose, maltose and mannitol discs (25 mg, Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. All the isolates tested here can ferment sucrose and produce acid. Isolates which were named as CK, WK, YK and SW/Cr can utilize all the nine sugars and produced acid. CK, WK and SW/Cr these three isolates were finally identified as the strains of the same species *K. oxytoca*. SW/Yel which is a Gram-positive isolate can ferment only sucrose among the used carbohydrates (Table 3.11).

Table 3.11 Isolates (N=9) showed various results on carbohydrate utilization test.

Bacterial Isolates	25 mg disc (Hi-Media)					20% solution			
	Sucrose	**Fructose**	**Galactose**	**Maltose**	**Mannitol**	**Glucose**	**Lactose**	**Arabinose**	**Xylose**
Type	disaccharide	ketonic monosaccharide	monosaccharide	disaccharide	sugar alcohol	monosaccharide	disaccharide	monosaccharide	monosaccharide
Molecular Formula	$C_{12}H_{22}O_{11}$	$C_6H_{12}O_6$	$C_6H_{12}O_6$	$C_{12}H_{22}O_{11}$	$C_6H_{14}O_6$	$C_6H_{12}O_6$	$C_{12}H_{22}O_{11}$	$C_5H_{10}O_5$	$C_5H_{10}O_5$
CK	+	+	+	+	+	+	+	+	+
WK	+	+	+	+	+	+	+	+	+
YK	+	+	+	+	+	+	+	+	+
CD	+	+	+	+	+	+	+	-	-
YD	+	+	+	+	+	+	-	-	-
CYD	+	+	+	+	+	+	-	-	-
SW/Cr	+	+	+	+	+	+	+	+	+
SW/Yel	+	-	-	-	-	-	-	-	-
GSH/Cr	+	+	+	+	+	+	+	-	-

3.3 Antibiotic susceptibility test

Isolates showed variable results however some of them were from same species with different strains. Isolate CK and WK showed more or less same results with a less exception while isolate SW/Cr was from the same species *K. oxytoca* but showed different type of sensitivity against some antibiotics. Among the nine isolates six showed resistance against bacitracin. All the isolates are more or less sensitive to gentamicin, neomycin, cephradine, doxycycline, tetracycline, ceftriaxone, ciprofloxacin, pefloxacin, mecillinam and nitrofurantoin (Table 3.12, Plate 3.15).

Table 3.12 Sensitivity or resistance showed by isolates (N=9) against different β-lactam antibiotics.

Class: β-lactam antibiotics	Name of the Antibiotics	Dose («g)	Inhibition zone (mm) for the isolates								
			CK	WK	YK	CD	YD	CYD	SW/ Cr	SW/ Yel	GSH /Cr
Aminoglycosides	Gentamicin	10	20	18	22	14	12	12	16	14	16
	Neomycin	30	16	12	26	16	08	12	13	13	14
Macrolides	Azithromycin	15	18	20	36	14	R	15	18	20	23
	Erythromycin	15	25	26	38	14	R	18	R	22	15
Penicillins	Ampicillin	10	12	8	25	R	13	25	09	22	R
Cephalosporins of 1st Generation	Cephradine	30	26	20	38	12	17	18	12	18	16
Tetracyclines	Doxycycline	30	12	12	20	15	16	18	13	22	16
	Tetracycline	30	16	10	18	18	18	20	18	18	20
Cephalosporins of 3rd Generation	Ceftriaxone	30	16	10	36	31	13	23	18	20	36
Quinolones/ Fluoroquinolones	Ciprofloxacin	5	13	18	24	15	14	24	20	18	26
	Pefloxacin	5	15	12	28	20	12	10	18	15	25
Amdinocillin	Mecillinam	25	08	10	12	14	11	12	08	10	11
Sulfonamides	Sulphamethoxazole/ Trimethoprim	25	R	R	28	10	14	12	16	18	16
Nitrofurantoin	Nitrofurantoin	300	12	14	20	18	10	10	16	16	20
Polypeptides	Bacitracin	10 units	R	R	R	R	11	16	R	16	R

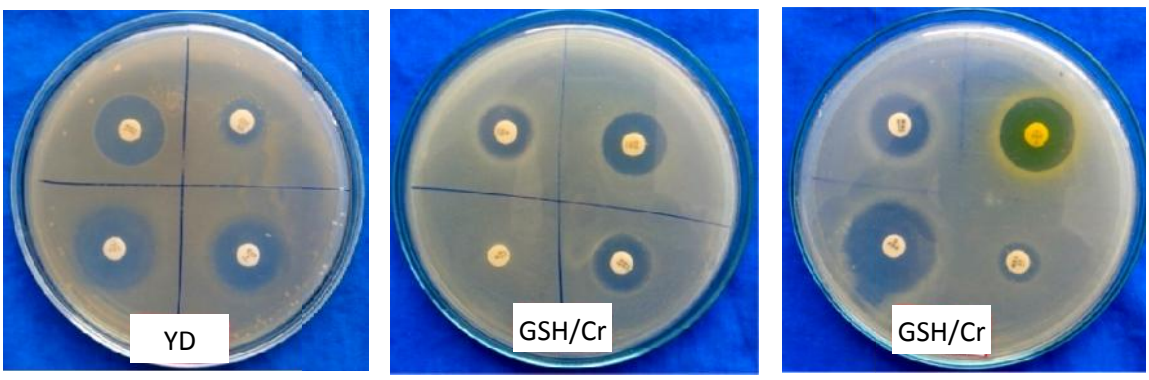


Plate 3.15 Antibiotic sensitivity pattern of some of the isolates.

3.4 Colony forming unit (CFU), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

LB broth suspension poured on to LB agar petri plates, spreaded and kept inverted at ~37°C for overnight. On the following day colonies were counted manually where numbers of the growing colonies were in between 30 to 300. The maximum dilution needed for the isolate YD and the lowest dilution was for the isolate SW/Yel (Table 3.13). The CFU/ml was in the range of 42-50×10^{10}, 39-48×10^{10}, and 37-43×10^{10} for the three isolates CK, WK, and SW/Cr respectively which were different strains of the same genus *K. oxytoca*. For the two isolates CD and GSH/Cr from the same genus *Aeromonas* CFU/ml were 34-41×10^{10} and 32-39×10^{10} respectively. CFU/ml was recorded as 65-71×10^{10} and 58-64×10^{10} respectively for the two isolates YD and CYD from the genus *Brevibacillus*. Isolate YK and SW/Yel formed 23-27×10^{10} and 11-16×10^{8} CFU/ml respectively.

MIC and MBC for the used two antibiotics ampicillin and tetracycline were determined through tube dilution method and results were recorded depending on tube turbidity. The suspension from the MIC tube was poured on to LB agar plate and kept inverted for overnight incubation at ~37°C. On the following day on the basis of colony formation results were noted and confirmed for MIC and MBC. Two isolates (CD and GSH/Cr) were found ampicillin resistant and MIC for this antibiotic ranged from 320-640 µg/ml and MBC was in the range of 320-1280 µg/ml. The MIC and MBC were ranged from 320-640 µg/ml and 320-1280 µg/ml respectively for the antibiotic tetracycline as all the isolates were sensitive to this (Table 3.13).

3.5 Optimizing growth of the isolates

The procaryotes exist in nature under an enormous range of physical conditions such as O_2 concentration, Hydrogen ion concentration (pH) and temperature. The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganism, most often a procaryote, and frequently an Archaeon. Applied to all microorganisms is a vocabulary of terms used to describe their growth (ability to grow) within a range of physical conditions. A thermophile grows at high temperatures, an acidophile grows at low pH, an osmophile grows at high solute concentration, and so on.

Optimization of bacteria is one of the major constraints in microbiological laboratories. It takes a series of work and difficulties hampered in many ways when there is a possibility or chance of contamination. On the basis of physiological and biochemical characteristics of the isolates five were chosen (CK, YK, CD, YD and SW/Yel) for optimization at different temperatures, pH and salinity. As the isolates were collected from the temperate region they showed their optimum growth in the range of temperature 28–30°C, pH 6.8–7.5 and salinity 1% (Figure 3.1-3.5). For all the biochemical tests pH were maintained at 7.2 (unless otherwise indicated) and pH 7.0 was found as suitable for all the isolates. All the five isolates were comfortable to grow at 1% salinity. At 37°C growth of the colonies were found as rapid but colonies overlapped when incubation period was more than 18 to 20 hours. They showed good growth with well isolated distinct colonies at 30°C. Gram-negative six isolates gave distinct, and well elevated and bright pigmented colonies when they were kept at 4°C in refrigerator. The Gram-positive isolates got pale when refrigerated but colonies became distinct.

Table 3.13 Colony forming unit (CFU), and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the isolates in two different antibiotics (N=9).

Isolates	CFU/ml	Ampicillin		Tetracycline	
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
CK	42-50×10^{10}	640	1280	320	640
WK	39-48×10^{10}	640	640	640	1280
YK	23-27×10^{10}	320	320	320	320
CD	34-41×10^{10}	Resistant	Resistant	320	640
YD	65-71×10^{10}	320	640	320	640
CYD	58-64×10^{10}	320	640	320	640
SW/Cr	37-43×10^{10}	640	640	640	640
SW/Yel	11-16×10^{8}	320	320	320	320
GSH/Cr	32-39×10^{10}	Resistant	Resistant	320	640

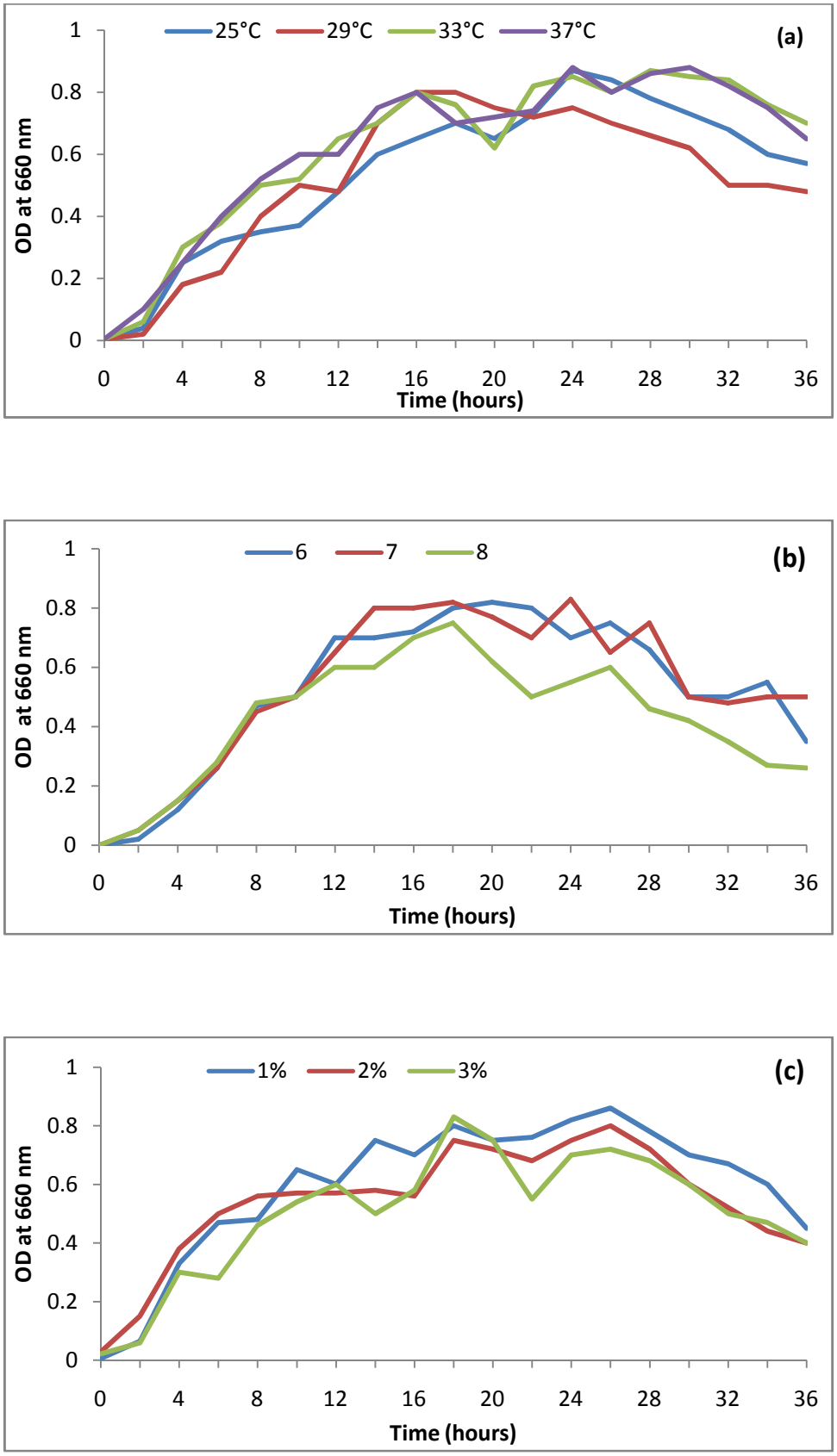


Figure 3.1 Growth characteristics of the isolate CK (creamy colony forming bacteria isolated from mucilage of diseased carp collected from Kakanhat area, Rajshahi) at different temperatures (a), pH (b) and salinity (c).

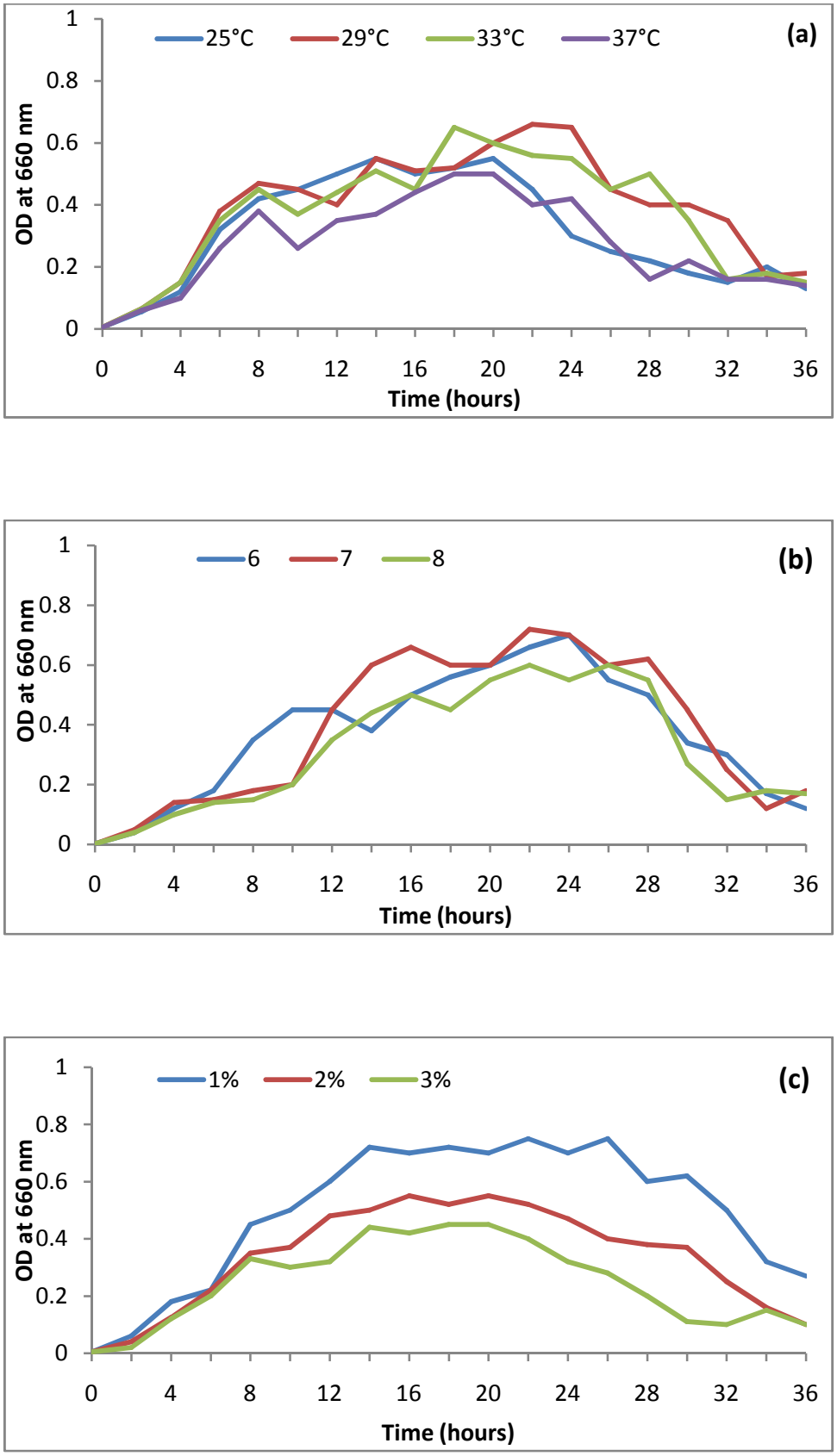


Figure 3.2 Growth characteristics of the isolate YK (yellow colony forming bacteria isolated from diseased carp collected from Kakanhat area, Rajshahi) at different temperatures (a), pH (b) and salinity (c).

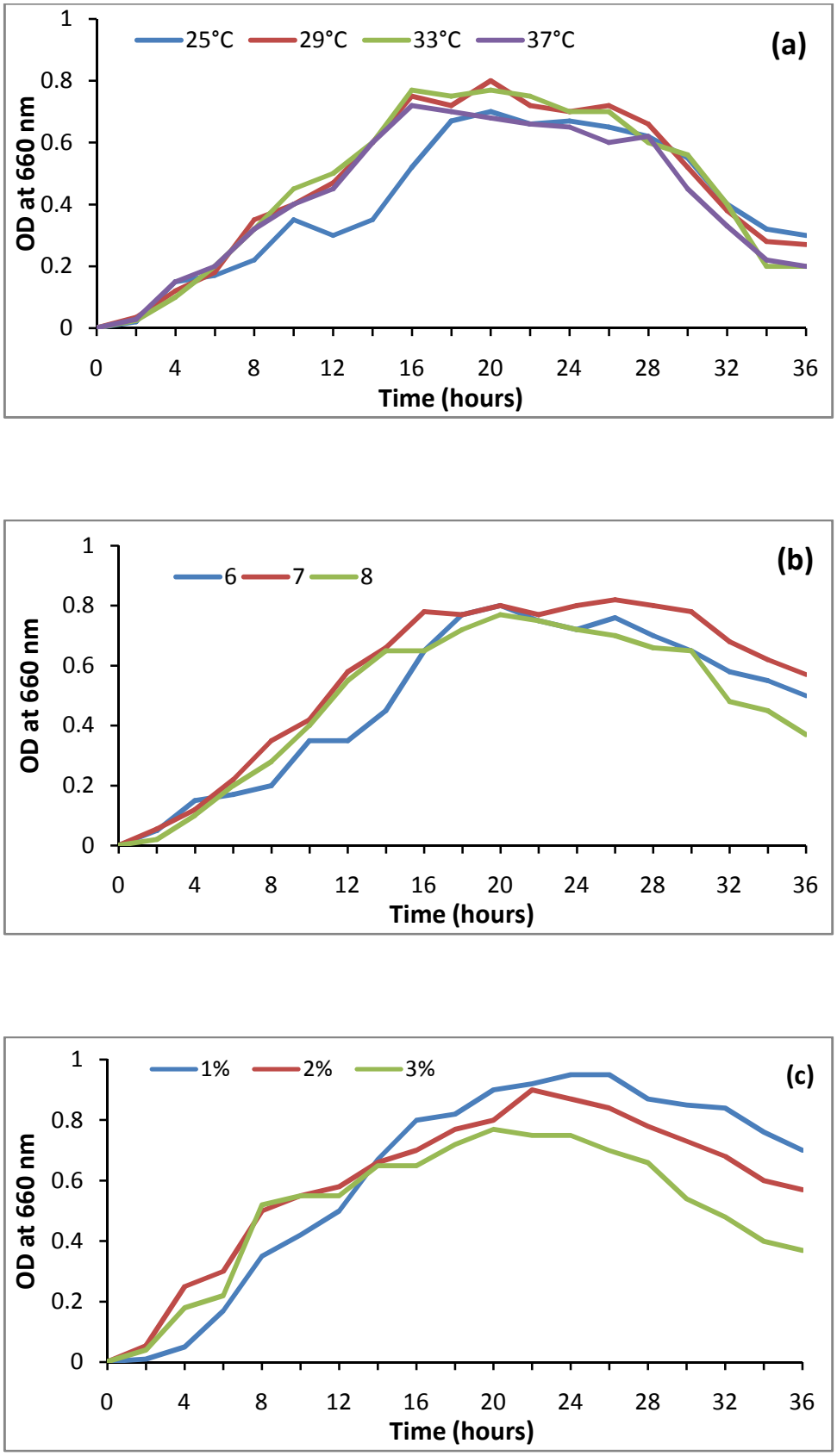


Figure 3.3 Growth characteristics of the isolate CD (creamy colony forming bacteria isolated from diseased carp collected from Darusha area, Rajshahi) at different temperatures (a), pH (b) and salinity (c).

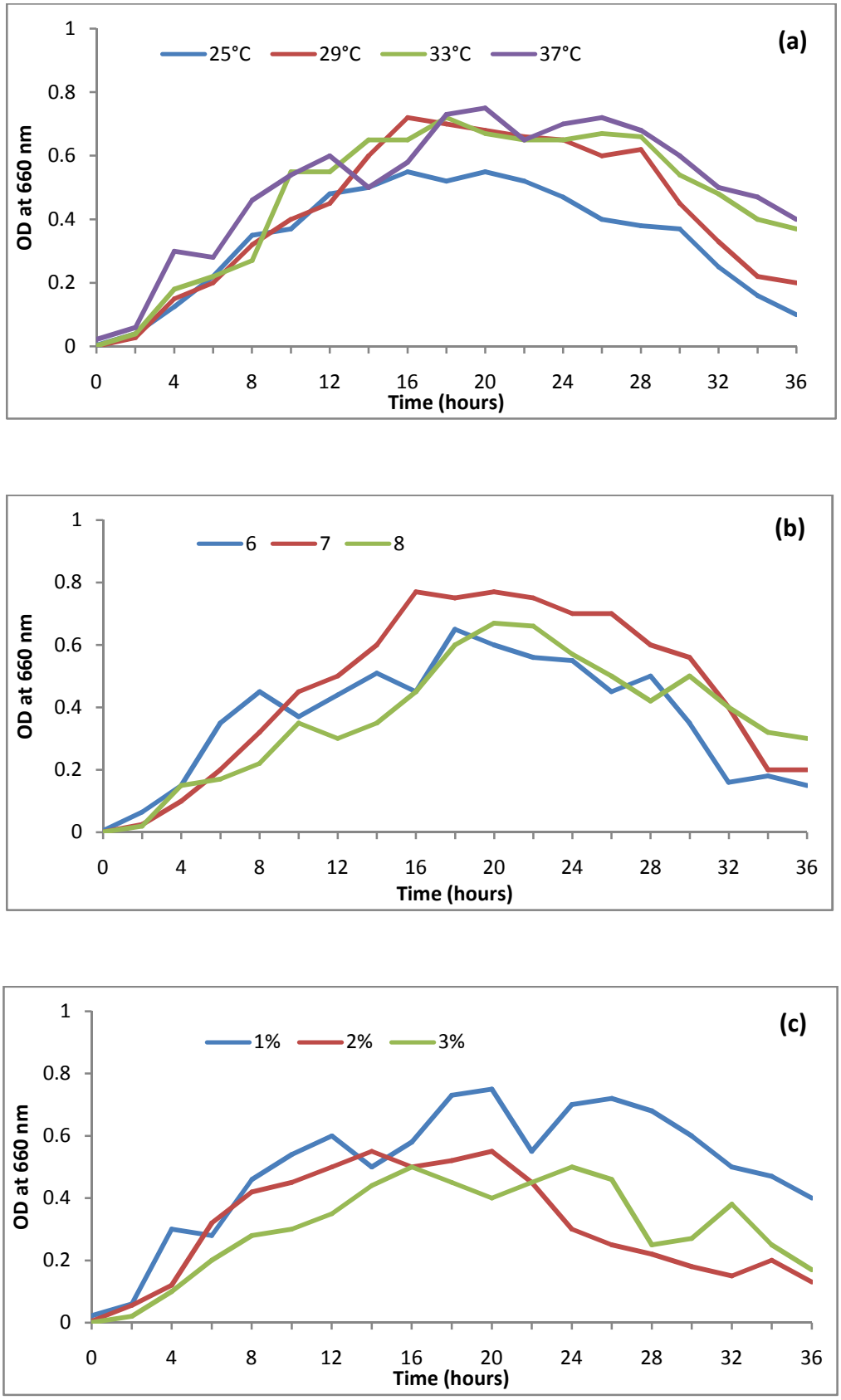


Figure 3.4 Growth characteristics of the isolate YD (yellow colony forming bacteria isolated from diseased carp collected from Darusha area, Rajshahi) at different temperatures (a), pH (b) and salinity (c).

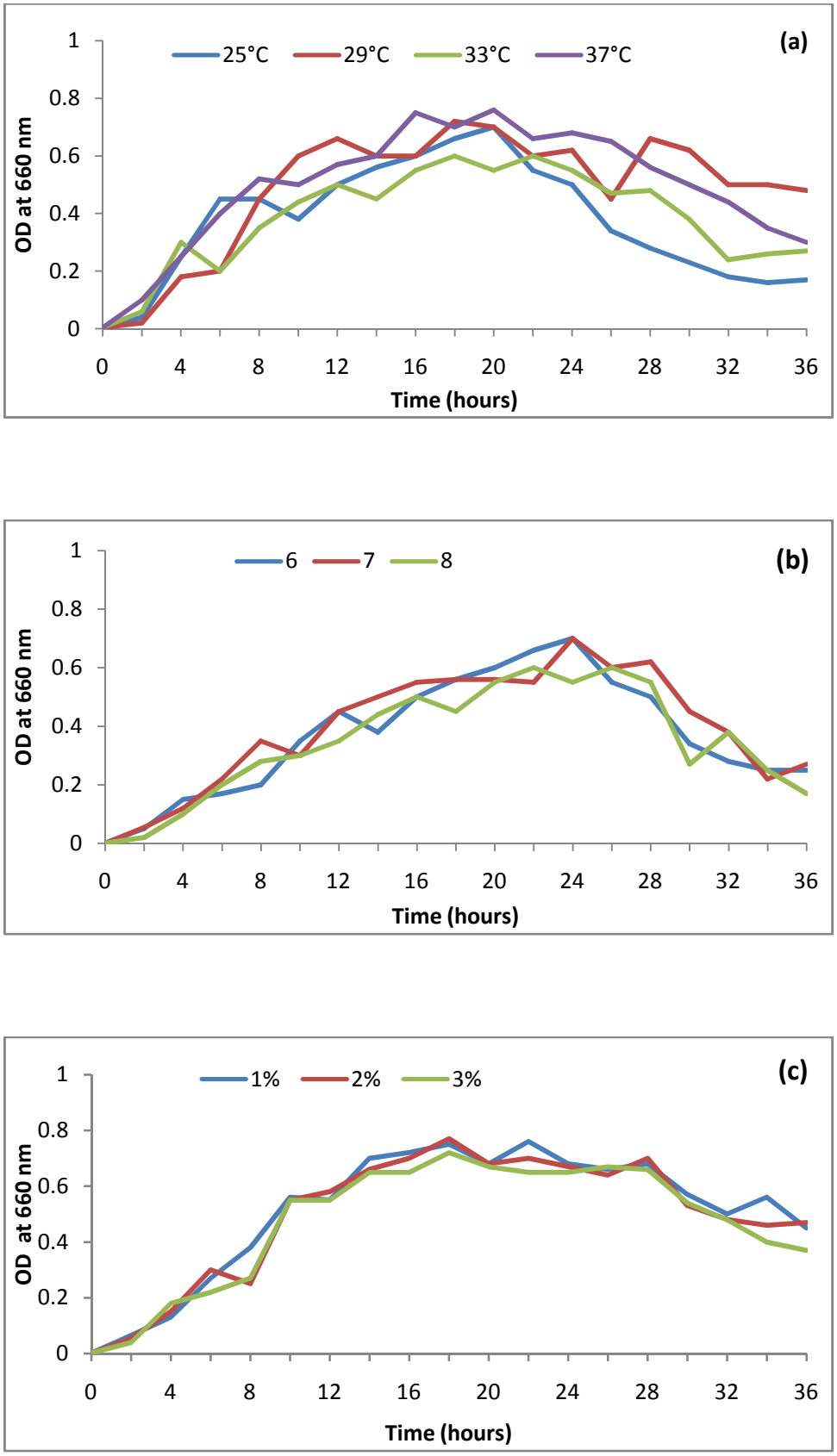


Figure 3.5 Growth characteristics of the isolate SW/Yel (yellow colony forming bacteria isolated from pond water collected from Ullapara, Sirajganj) at different temperatures (a), pH (b) and salinity (c).

3.6 Molecular identification

Genomic DNA extraction and gel electrophoresis: Genomic DNA from the 18 hours old broth culture of the nine isolates was extracted and gel electrophoresis was carried out to confirm its quality (Figure 3.6). We found a bright single band with high molecular weight and less smearing which was seen on top of the 10 kb band of the 1kb plus DNA ladder indicative of good quality DNA. We used this genomic DNA for our further molecular biology experiments.

We initially diluted the genomic DNA (1:4 ratios) and used 1µl of this diluted genomic DNA for PCR. We used five different primers in following combination to find out the best pair of primers to amplify the 16S rRNA gene: 8F–1492R, 8F–806R, 8F–907R, 515F–1492R, 515F–806R, and 515F–907R. Each and every PCR products were run through 1.0% agarose gel electrophoresis and visualized under UV-transilluminator. The combination of 8F–806R and 8F–1492R gave the more contrast single band in between 700–800 bp in comparison to 1kb plus DNA ladder (Figure 3.7).

Subsequently, PCR was carried with an increased volume (50 µl) using 8F–806R. PCR product was purified using TIANquick midi purification kit and prepared for sequencing PCR. As it was the objective to sequence the clone only from one end of the gene we further carried out PCR using the purified PCR product as template and one primer each time. Among the forward (8F) and reverse (806R) primers, only 8F generated brilliant single band and thus help us to decide to sequence our PCR product using 8F primer (Figure 3.8). Before big dye cycle sequencing using Big Dye terminator Version 3 (BD-V3) the purity and concentration of the purified PCR products were measured as it is needed for dilution. The results are presented in Table 3.14 for each and every isolate. The sequencing was carried out in an ABI Prism 3130 genetic analyzer and the obtained sequences are shown in Appendix V – XIII in FASTA format.

For a more precise classification, a sequence analysis of the 16S rRNA was carried out. The BLAST search results for the partial sequences of the 16S rRNA gene of the isolates revealed that three isolates CK, WK and SW/Cr are from the same species *K. oxytoca* with different strains. They had 98, 99 and 99% similarity with that of *K. oxytoca*, with sequence ID gbJX848325.1, gbGU361112.1 and gbHQ683946.1 respectively; as such, the three isolates were designated as *K. oxytoca* (Table 3.15). The third isolate YK found as *Pantoea* sp. with 84% similarity with gbJN853256.1. The fourth and ninth isolates CD and GSH/Cr bearing 97 and 98% similarity with gbKC916744.1 and FJ464586.1 respectively with different strains of *Aeromonas*. Fifth and sixth isolates (YD and CYD) having almost same physiological and biochemical

characteristics with some exception found with 99 and 91% similarity with gbJQ229800.1 and dbjAB491169.1 respectively of different strains of the genus *Brevibacillus* under the comparatively newly described family Paenibacillaceae (Table 3.15). The isolate SW/Yel was the only member from the family Bacillaceae which got 99% homogeneity with the genus *Bacillus* bearing ID gbJQ660682.1 (Table 3.15).

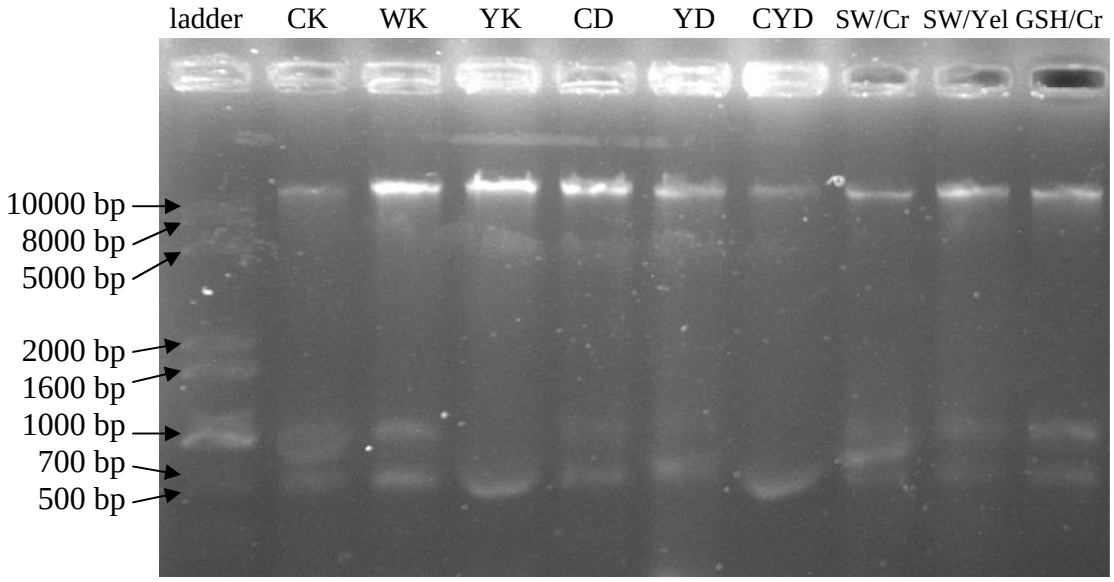


Figure 3.6 Genomic DNA of nine (9) isolates of the bacteria obtained from diseased carp fish using TIANamp Bacteria DNA Kit. DNA was run on 1% agarose gel containing Ethidium bromide. Lane 1: Tiangen 1 kb Plus DNA ladder, lane 2-10: isolates 1-9.

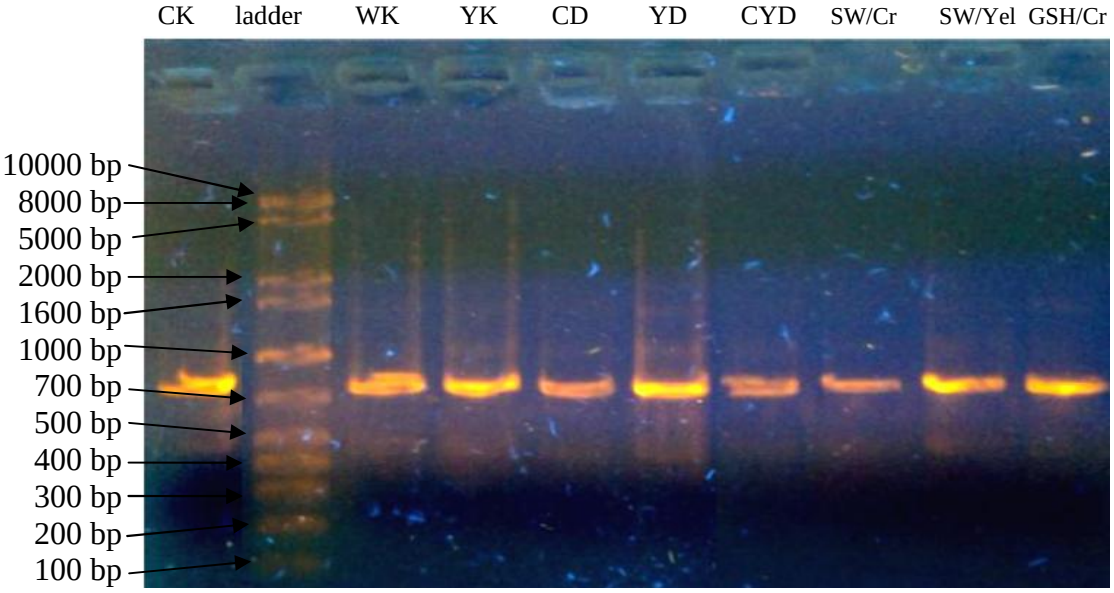


Figure 3.7 PCR products of nine (9) isolates of the bacteria obtained from diseased carp fish using 8F and 806R primers. PCR product was run on 1% agarose gel containing Ethidium bromide. Lane 1: Isolate CK, lane 2: Tiangen 1 kb Plus DNA ladder, lane 3-10: isolates WK, YK, CD, YD, CYD, SW/Cr, SW/Yel and GSH/Cr.

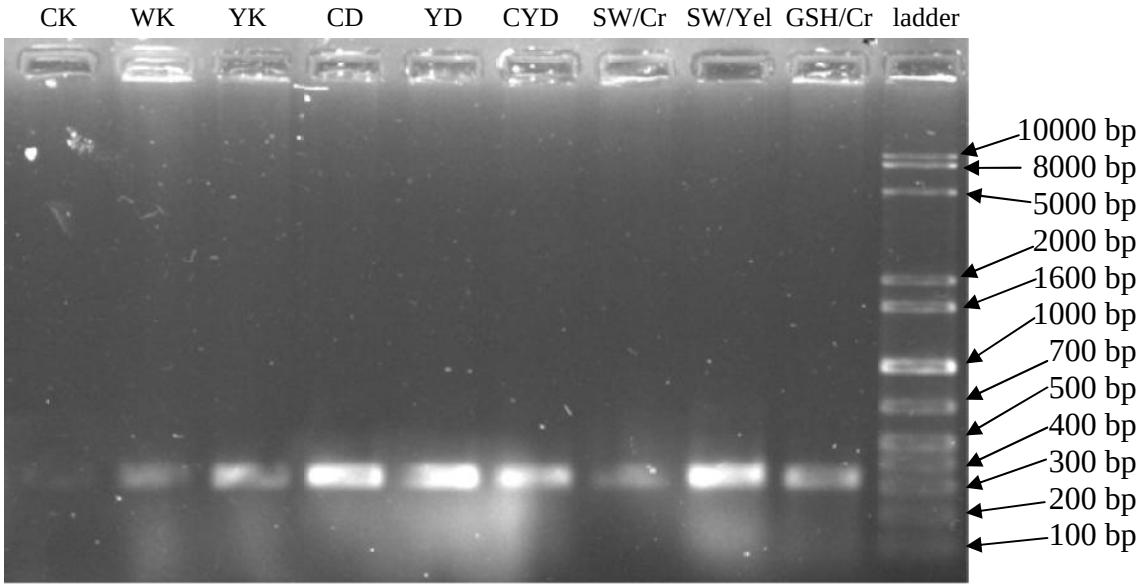


Figure 3.8 Purified PCR products using 8F primer generated brilliant single band of nine (9) isolates of the bacteria obtained from diseased carp fish. PCR product was run on 1% agarose gel containing Ethidium bromide. Lane 1-9: isolates 1-9 (CK, WK, YK, CD, YD, CYD, SW/Cr, SW/Yel and GSH/Cr), lane 10: Tiangen 1 kb Plus DNA ladder.

Table 3.14 Purity and concentration of the isolates (N=9) with dilution factor needed for big dye cycle sequencing.

Isolates	Purity (260/280 nm)	Concentration (ng/µl)	Dilution factor
CK	2.25	51.2	4 × d
WK	2.20	76.4	6 × d
YK	2.10	72.5	6 × d
CD	2.23	72.6	6 × d
YD	1.85	100.8	8 × d
CYD	1.87	89.5	8 × d
SW/Cr	2.44	49.3	4 × d
SW/Yel	2.11	79.5	6 × d
GSH/Cr	2.51	39.3	3 × d

Table 3.15 Isolates with 16S rDNA sequence match results found in NCBI database.

Isolates	Resembles with NCBI BLASTn suite			Family
	1st matching	Identities (%)	Sequence ID	
CK	*Klebsiella oxytoca*	728/742 (98%)	gbJX848325.1	Enterobacteriaceae
WK	*Klebsiella oxytoca*	737/743 (99%)	gbGU361112.1	Enterobacteriaceae
YK	*Pantoea* sp.	534/636 (84%)	gbJN853256.1	Enterobacteriaceae
CD	*Aeromonas* sp.	732/758 (97%)	gbKC916744.1	Enterobacteriaceae
YD	*Brevibacillus borstelensis*	734/738 (99%)	gbJQ229800.1	Paenibacillaceae
CYD	*Brevibacillus borstelensis*	685/751 (91%)	dbjAB491169.1	Paenibacillaceae
SW/Cr	*Klebsiella oxytoca*	737/746 (99%)	gbHQ683946.1	Enterobacteriaceae
SW/Yel	*Bacillus* sp.	751/755 (99%)	gbJQ660682.1	Bacillaceae
GSH/Cr	*Aeromonas veronii* bv. sobria	750/763 (98%)	FJ464586.1	Enterobacteriaceae

3.7 Identified isolates and their distinctiveness

***Aeromonas* sp. (isolates CD and GSH/Cr):** Cultures comprise Gram-negative enteric, motile fermentative straight rods, which produce indole, oxidase positive but catalase negative, did not reduce sulphur and grow in 0-3% (w/v) sodium chloride, at 4-42°C and at pH 6.7 to 9.0. On non-selective media, cream, round, umbonate, entire colonies of 2-3 mm diameter develop within 24h at 37°C. The methyl red test is positive, but not so the Voges-Proskauer reaction. Unable to ferment lactose and utilize citrate. Acid is produced from sucrose, fructose, galactose, maltose, mannitol, glucose (acid and gas), lactose but not from arabinose and xylose. Urea was not degraded by the two isolates (CD and GSH/Cr). Susceptibility has been recorded to gentamicin, neomycin, azithromycin, cephradine, doxycycline, tetracycline, ceftriaxone, ciprofloxacin, pefloxacin, mecillinam, sulphamethoxazole/ trimethoprim, erythromycin, nitrofurantoin, but not to ampicillin and bacitracin. Colony forming units of the two isolates determined as in the range of $32\text{-}41\times10^{10}$ per ml on nutrient agar plate while MIC and MBC were recorded as 320 and 640 µg/ml on tetracycline respectively for both of the isolates.

***Bacillus* sp. (isolate SW/Yel):** Using nutrient agar plates with an incubation temperature of ~37°C, yellow pigmented, rough, opaque colonies obtained. Yellow coloured colonies were obtained on TSA after 24h incubation at 28°C. These colonies comprise non motile, fermentative Gram-positive rods of 1–4 mm in length. The cells can grow up to 15-42°C and can tolerate refrigerated temperature (4°C) where growing colonies became distinct with their colour and shape. No signs of growth on MacConkey agar and Simmons citrate agar as they are Gram-positive. The isolate showed catalase activity but not oxidase, unable to catabolize sugar and peptone on TSI agar medium and hydrolyze urea. The methyl red test was negative, while the Voges-Proskauer reaction was positive. Acid has been produced from only sucrose among the nine used sugars. The isolate was susceptible to all the antibiotics used. The isolate was determined as low colony grower with CFU in the range of $11\text{-}16\times10^{8}$ per ml on nutrient agar plate while MIC and MBC were recorded as 320 µg/ml on ampicillin and tetracycline.

***Brevibacillus* sp. (isolates YD and CYD):** On nutrient agar plates after a long process of sub-culturing to obtain pure colony isolate YD and CYD gave large, circular, flat, opaque, creamy-orange coloured colony where there was a little difference on their colour. YD gave brighter colony than that of CYD as they are denoted as yellow and creamy-yellow. Both of them were Gram-positive short rod but YD sometimes played as Gram-variable and both of them were unable to grow on MacConkey agar and Simmons citrate agar; hence confirmed as Gram-positive. Both of them were weak positive on catalase activity and fermentative but YD found as oxidase negative while CYD was oxidase positive. On TSI agar slant they fermented sugars but did not grow in the anaerobic area of the butt. They were found as weak motile on SIM media but no evidence was seen on MIU media. As YD isolate were noted as Gram-variable it showed another variable result for urea hydrolysis, methyl red test, and Voges-Proskauer reaction. YD can hydrolyze urea but CYD cannot, YD was negative for both MR-VP but CYD was negative on MR and positive on VP. Both of the isolates showed same characters on acid production from sugars. They produced acid from sucrose, fructose, galactose, maltose, mannitol and glucose but not from lactose, arabinose and xylose. Isolate CYD showed sensitivity for all the used antibiotic discs but YD was resistant over macrolides (azithromycin and erythromycin). YD showed more growth for CFU than CYD and determined as 65-

71×10^{10} and $58\text{-}64\times10^{10}$ per ml while MIC and MBC were recorded as 320 and 640 µg/ml respectively for both the *Brevibacillus* sp.

***Klebsiella oxytoca* (isolates CK, WK and SW/Cr):** Culture comprises fermentative Gram-negative rods that produce catalase and indole but not H_2S, and oxidase negative. The three isolates (CK, WK and SW/Cr) showed motility on SIM media but only isolate SW/Cr was motile in MIU media. Isolate CK and WK were lactose fermenters while SW/Cr was not though they all showed growth on MAC agar and SC agar. All the three were gas producer and exhibited acidic fermentation of sugars in TSI agar and can hydrolyze urea. The first two were MR positive and VP negative while SW/Cr showed different results for VP. Produce acid from used all the sugars i.e. sucrose, fructose, galactose, maltose, mannitol, glucose, lactose, arabinose and xylose. Antibiotic susceptibility pattern was the same for CK and WK as they showed resistance against Sulphamethoxazole/ Trimethoprim and Bacitracin. SW/Cr showed resistance on erythromycin and bacitracin. On an average per ml of LB broth, $37\text{–}54\times10^{10}$ colonies were produced by the isolates. MIC and MBC values for isolate CK recorded as 640 and 1280, 320 and 640 µg/ml for ampicillin and tetracycline respectively. MIC was recorded as 640 and 640, and MBC was 640 and 1280 µg/ml for ampicillin and tetracycline respectively for the isolate WK. All the values were 640 µg/ml for both the antibiotics for the isolate SW/Cr.

***Pantoea* sp. (isolate YK):** Pale yellow colonies on nutrient agar media were obtained after 30–36 hours of incubation at ~37°C. Culture comprises transparent and viscid colonies, non-motile fermentative Gram-negative rods, lactose non-fermenter, catalase and oxidase negative and citrate utilized by the YK isolate. The MR-VP reactions were positive but not reduce H_2S and produce indole. Acid was produced from used all the used sugars. Variant results were noted on urea hydrolysis. In comparison to other eight isolates, slow growing and less number of colonies per ml of LB broth on LB agar was determined and the value is $23\text{–}27\times10^{10}$. All but bacitracin showed sensitivity while MIC and MBC values were recorded for both the antibiotics tested as 320 µg/ml.

3.8 Phylogenetic analysis of the isolated bacteria

16S ribosomal RNA gene partial sequences of nine isolates were analyzed using similarity search tool BLAST. This indicated high degree of similarity (84 to 99%) with annotated sequences of around 100. Phylogenetic tree based on 16S rDNA sequences

were constructed using neighbor joining method over a panel of 25 strains which exhibited maximum similarity.

Analysis of the phylogram for the isolate CK and the similarity matrix revealed three main branches between the new isolate of *K. oxytoca* (Figure 3.9). The similarity between species in this group was greater than or equal to 98% (Table 3.15). The distance between isolate CK and the first match gbJX848325.1 was the same (0.003) and it revealed that the isolate was nearer from their origin.

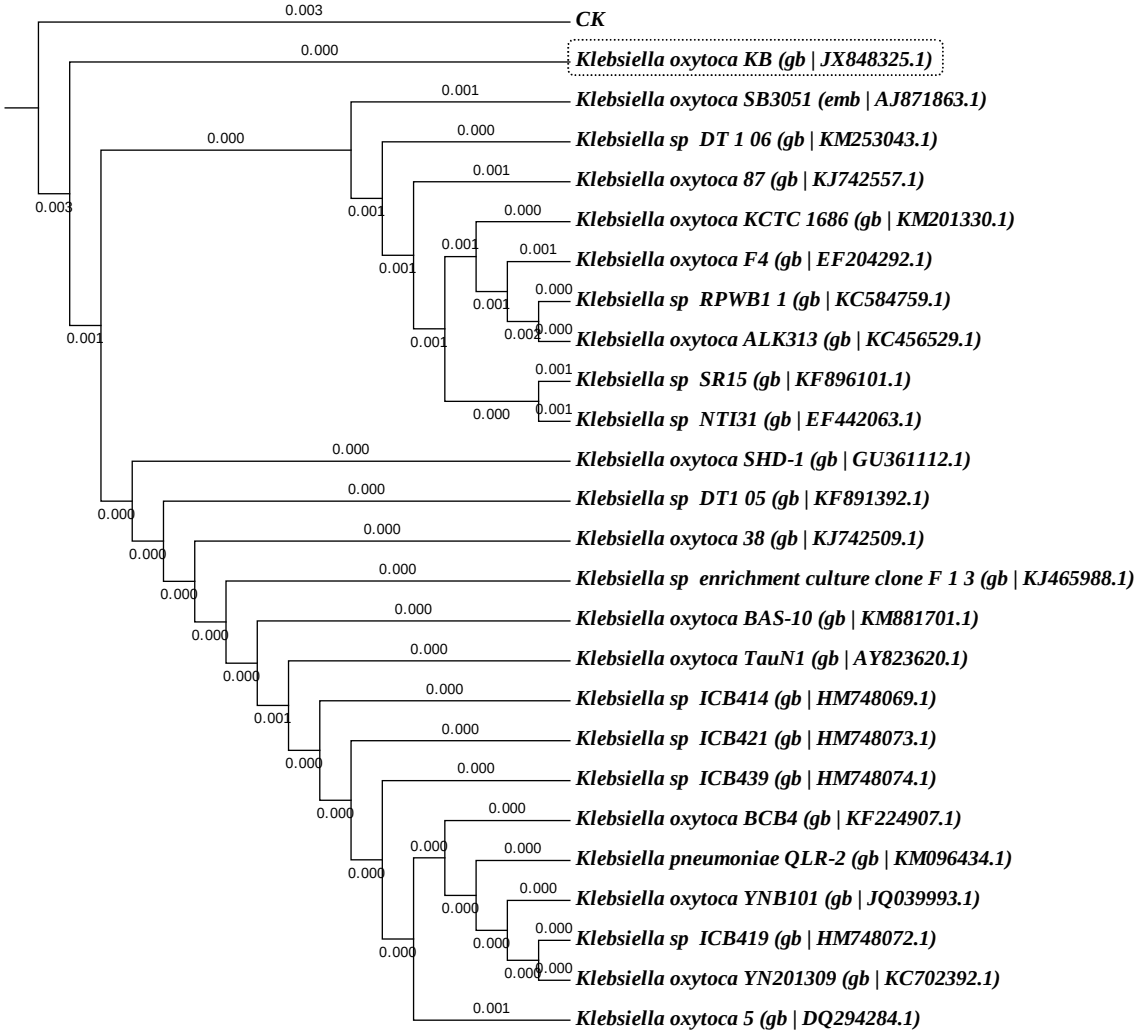


Figure 3.9 Unrooted phylogenetic tree for the isolate CK (creamy colony forming bacteria isolated from mucilage of diseased carp collected from Kakanhat area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first match.

Analysis of the phylogram for the second isolate WK (Figure 3.10) and the similarity matrix revealed that the new isolate was far away from the first match (Table 3.15) though it resembles 99% similar identity with the strain identity gbGU361112.1. The similarity between species in this group was greater than or equal to 99%. The first match was 0.003 distant from the source node while isolate WK was far-away double (0.006).

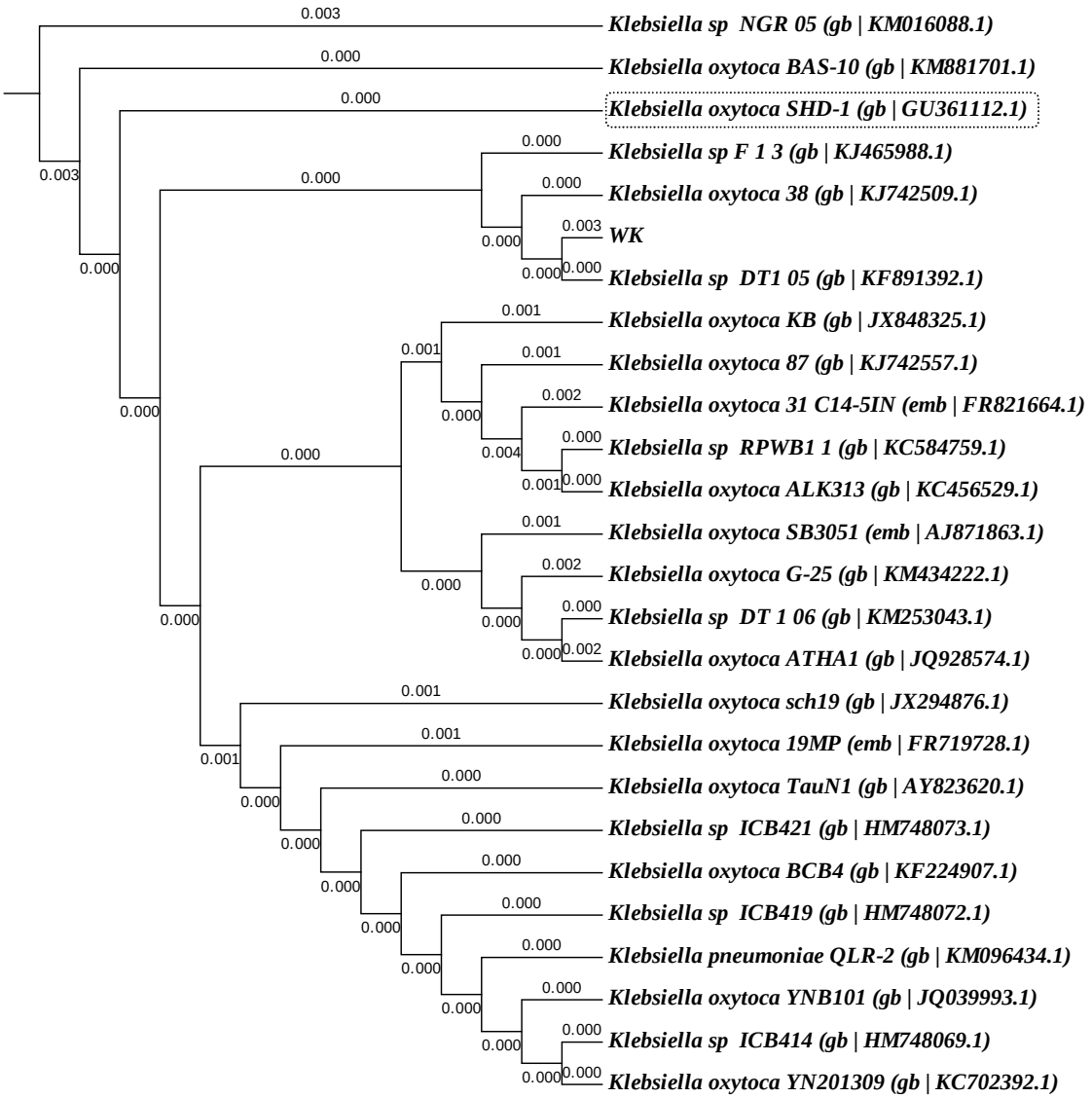


Figure 3.10 Unrooted phylogenetic tree for the second isolate WK (white colony forming bacteria isolated from diseased carp collected from Kakanhat area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first match.

Analysis of the phylogram for the isolate YK and the similarity matrix revealed two main branches between the new isolate of *Pantoea* (Figure 3.11). The similarity between species in this group was greater than or equal to 84%. The distance from the origin was 0.197 (0.070 + 0.007 + 0.008 + 0.009 + 0.103) and between isolate YK and the first match gbJN853256.1 (Table 3.15) was 0.068 and it revealed that the isolate was far away from origin. Not only that, in the list of first 25 matches there were another genus which shows some level of similarity with the isolate.

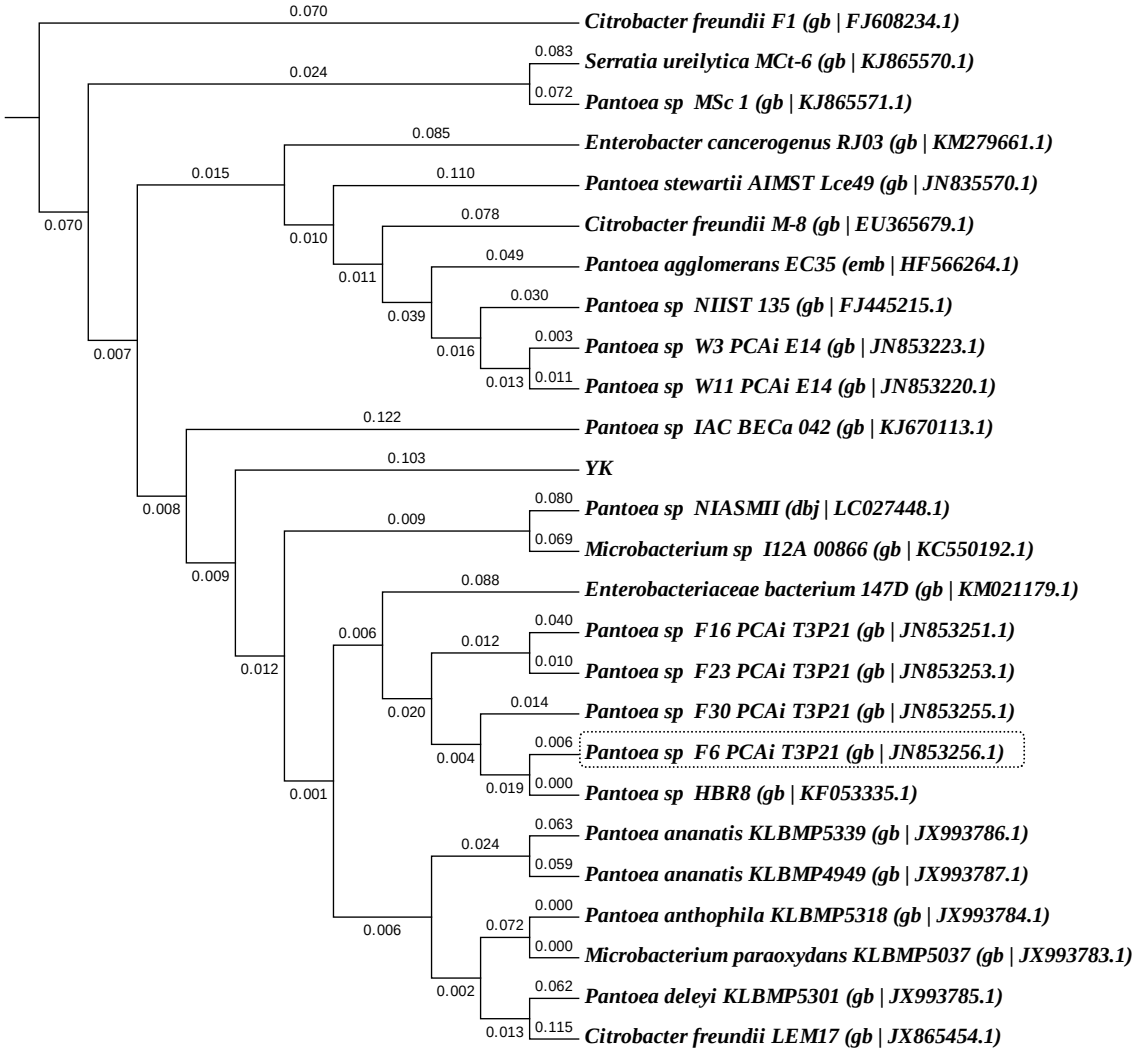


Figure 3.11 Unrooted phylogenetic tree for the isolate YK (yellow colony forming bacteria isolated from diseased carp collected from Kakanhat area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first match.

Phylogram analysis of the isolate CD showed two main branches where CD was only one in one branch and other 25 matches positioned in another (Figure 3.12). From the origin isolate CD was positioned at 0.007. The distance matrix between the isolate of *Aeromonas* and the first match gbKC916744.1 (Table 3.15) was calculated as 0.002. The similarity between species in this group was greater than or equal to 97%.

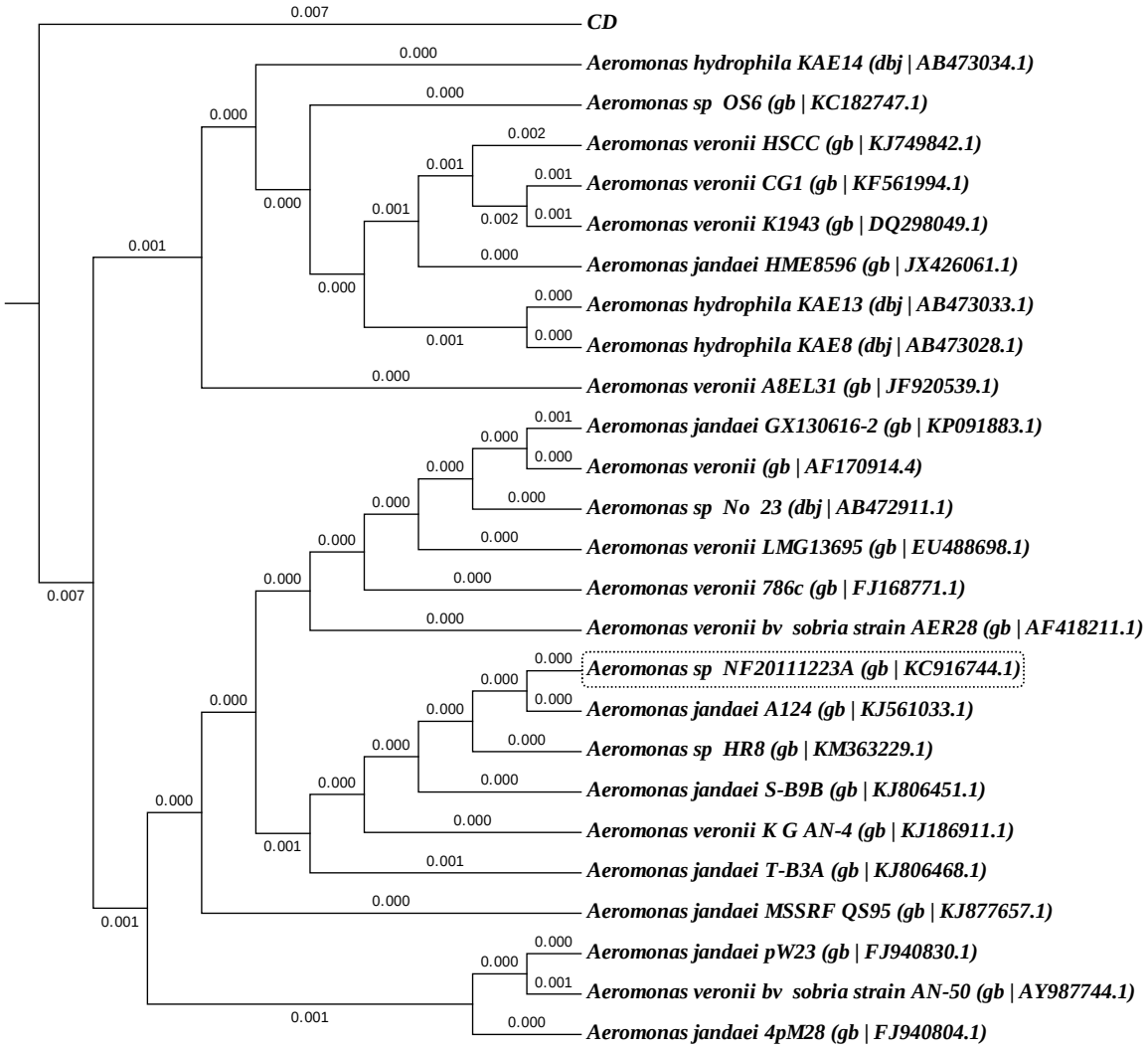


Figure 3.12 Unrooted phylogenetic tree for the isolate CD (creamy colony forming bacteria isolated from diseased carp collected from Darusha area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first match.

Phylogram analysis of the isolate YD showed two main branches where the first match (gbJQ229800.1) was distanced 0.0018 from its origin while YD was distanced 0.0019 (Figure 3.13). The similarity between species in this group was greater than or equal to 99% (Table 3.15).

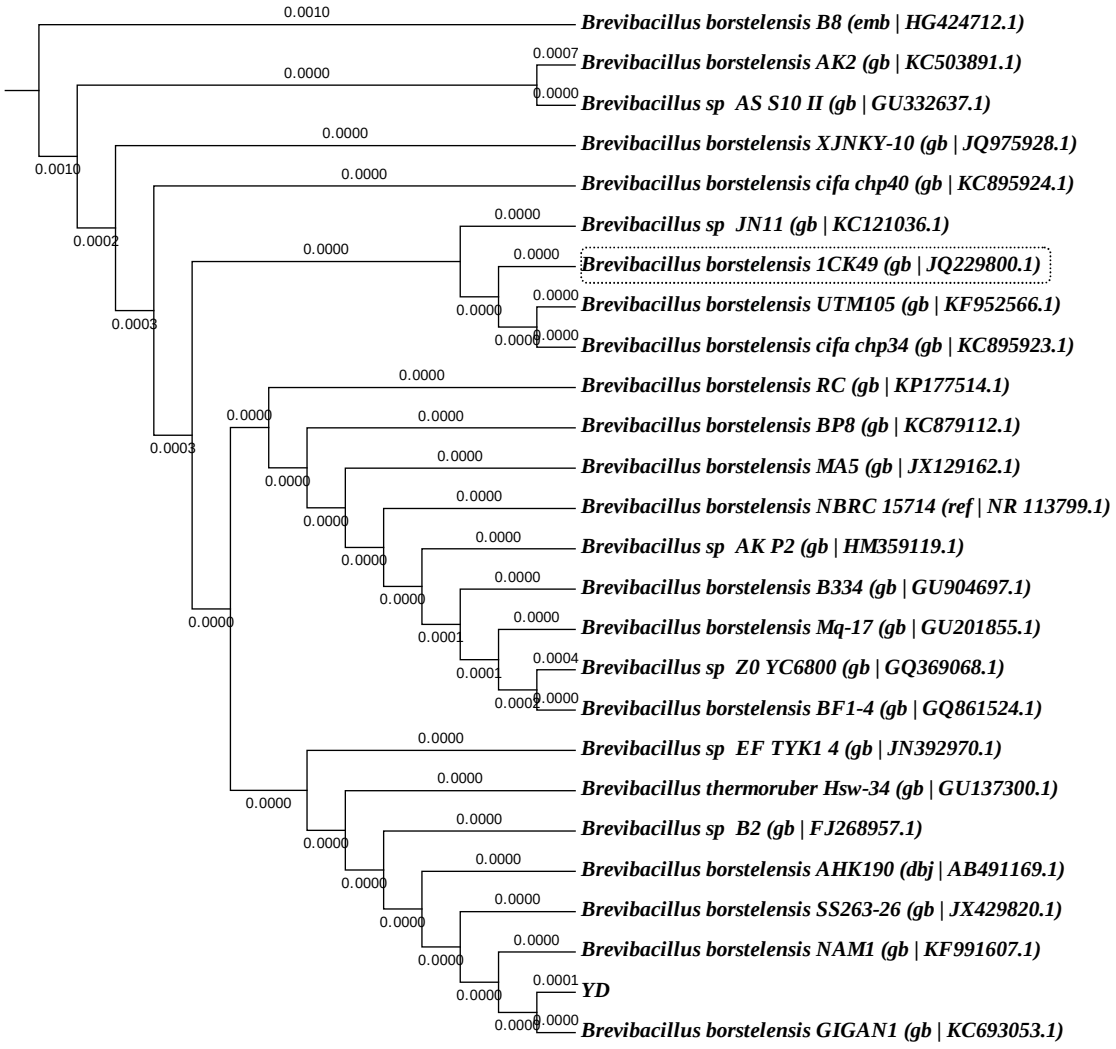


Figure 3.13 Unrooted phylogenetic tree for the isolate YD (yellow colony forming bacteria isolated from diseased carp collected from Darusha area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first match.

Analysis of the phylogram for the isolate CYD (Figure 3.14) and the similarity matrix revealed two main branches between the isolate of *B. borstelensis*. The similarity between species in this group was greater than or equal to 91%. The distance between isolate CYD and the first match dbjAB491169.1 (Table 3.15) was the same (0.037) and it revealed that the isolate was nearer from their origin.

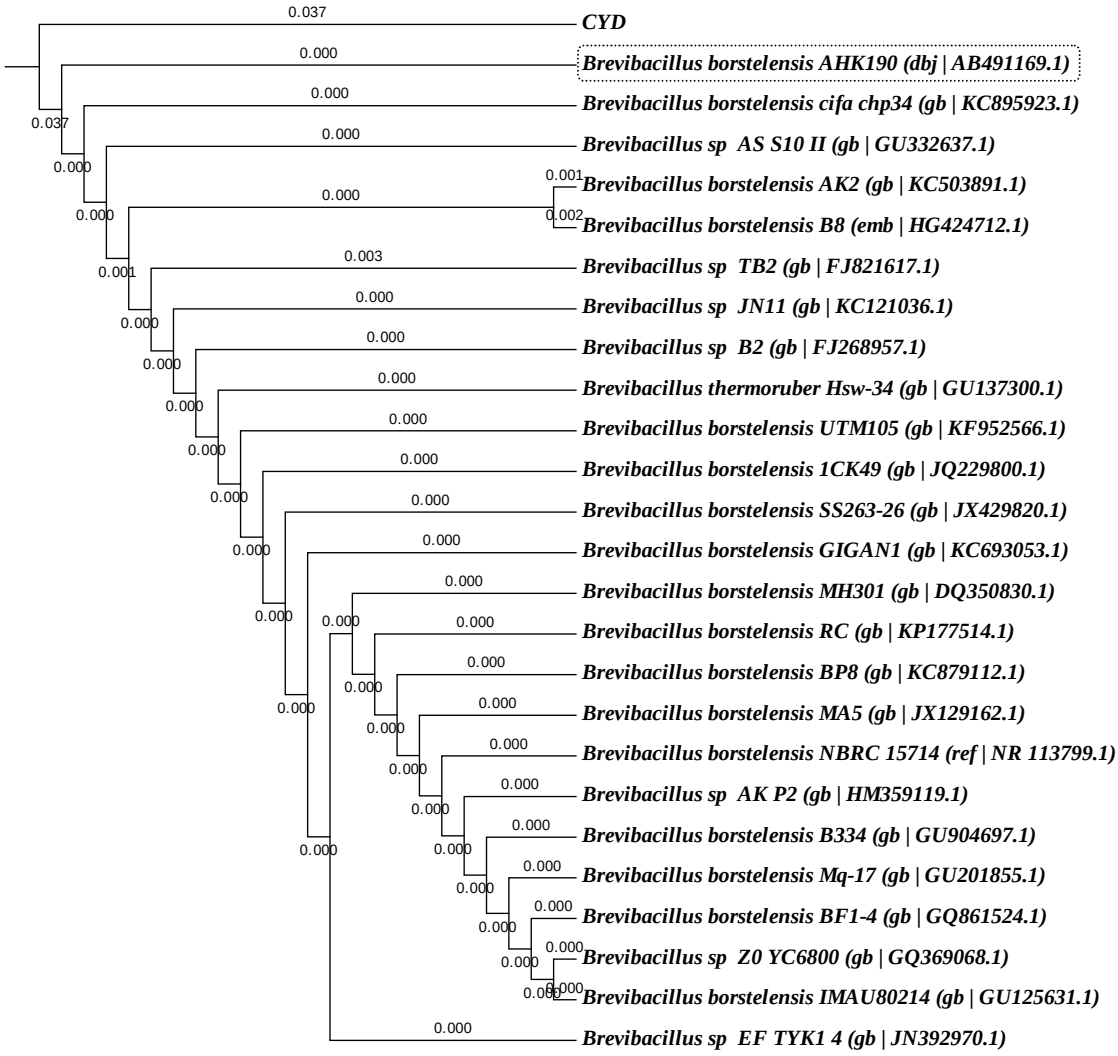


Figure 3.14 Unrooted phylogenetic tree for the isolate CYD (creamy-yellow colony forming bacteria isolated from diseased carp collected from Darusha area, Rajshahi) with distance with first 25 matches. Rectangular box indicates the first matchUnrooted phylogenetic tree for the isolate CYD with distance.

Analysis of the phylogram for the isolate SW/Cr and the similarity matrix revealed two branches (Figure 3.15). Homogeneity was established as 99% with the first match *K. oxytoca* gbHQ683946.1 (Table 3.15), though there were different strains of *Enterobacter* listed in the first 25 matches. The distance from the origin was 0.0057 for SW/Cr and 0.0077 for the first match of *K. oxytoca* and it revealed that the isolate as well as first match was far away from origin.

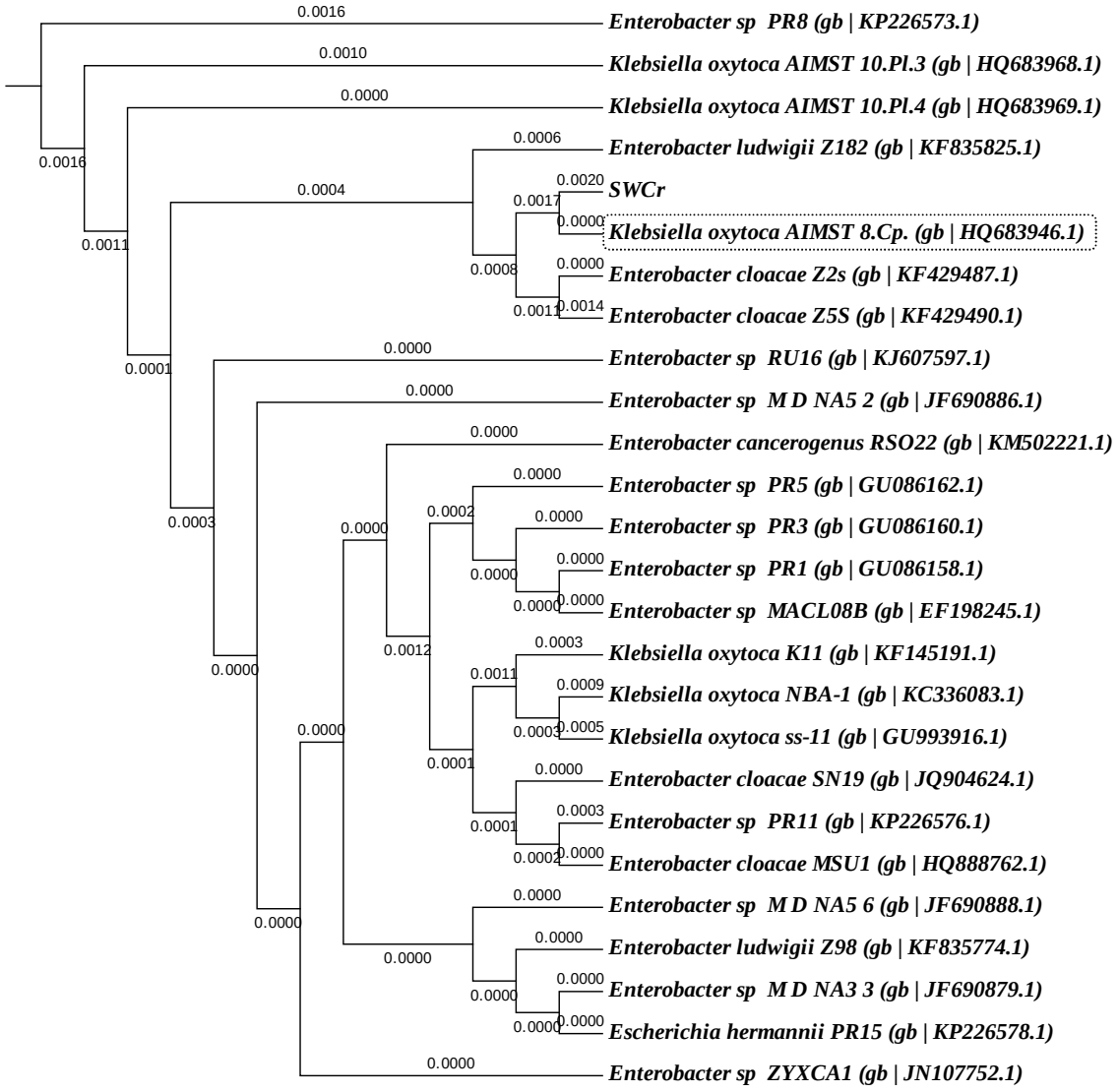


Figure 3.15 Unrooted phylogenetic tree for the isolate SW/Cr (creamy colony forming bacteria isolated from pond water sample collected from Ullapara, Sirajganj) with distance with first 25 matches. Rectangular box indicates the first match.

Phylogram for the isolate SW/Yel and the first 25 sequences from the NCBI database revealed two main branches and all the strains from the same *Bacillus* sp. (Figure 3.16). The similarity between species in this group was greater than or equal to 99% (Table 3.15) as all were from the same genus. The distance calculated as 0.00019 and 0.00053 from the origin for the isolate SW/Yel and the first match respectively (gbJQ660682.1).

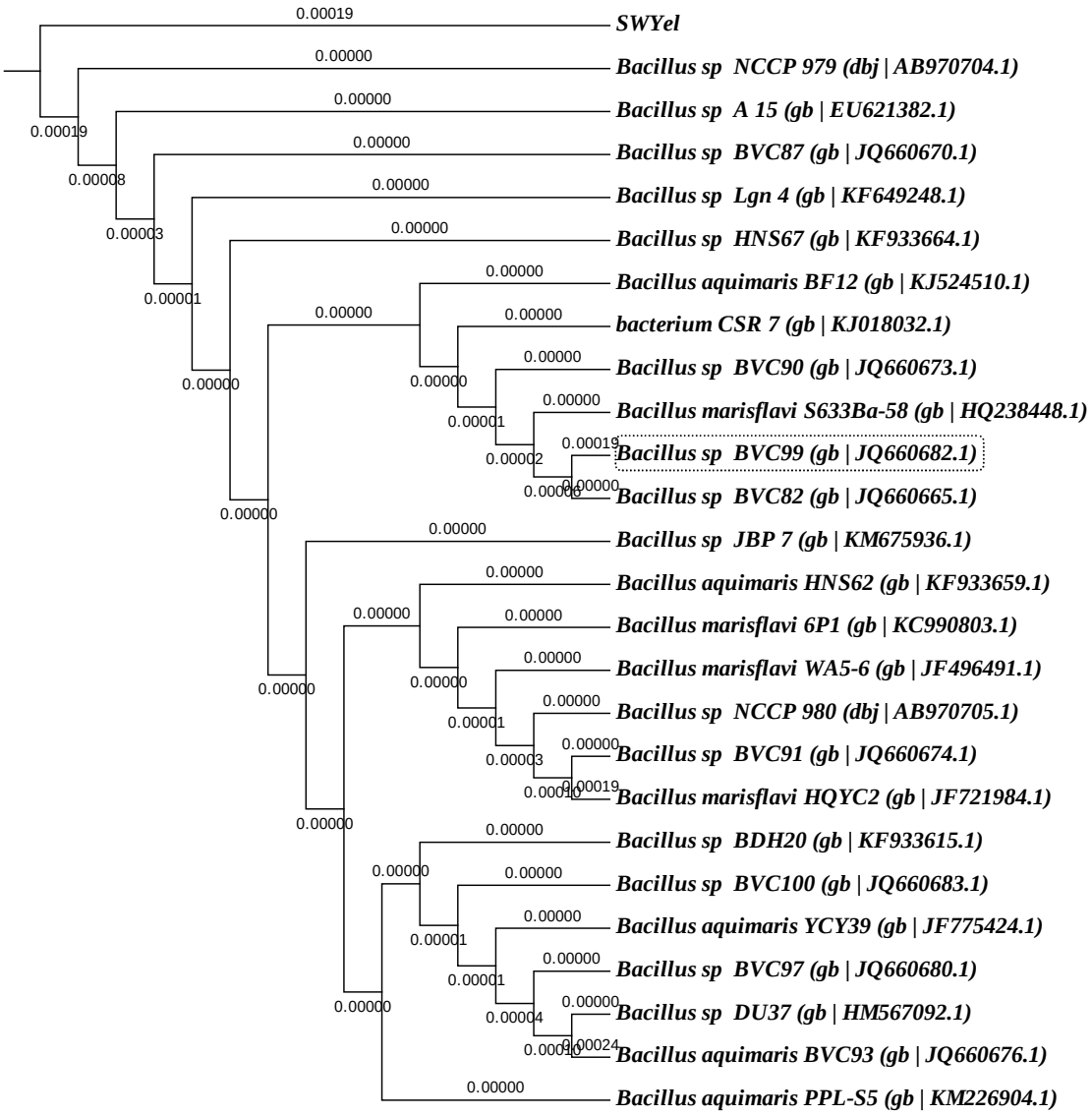


Figure 3.16 Unrooted phylogenetic tree for the isolate SW/Yel (yellow colony forming bacteria isolated from pond water sample collected from Ullapara, Sirajganj) with distance with first 25 matches. Rectangular box indicates the first match.

Phylogram of the isolate GSH/Cr and the similarity matrix revealed three main branches where the isolate placed in one branch with 0.003 distances where second was placed at 0.005 and the first match *A. veronii* bv. sobria with 98% homogeneity (Table 3.15) at distance 0.009 from their origin (Figure 3.17).

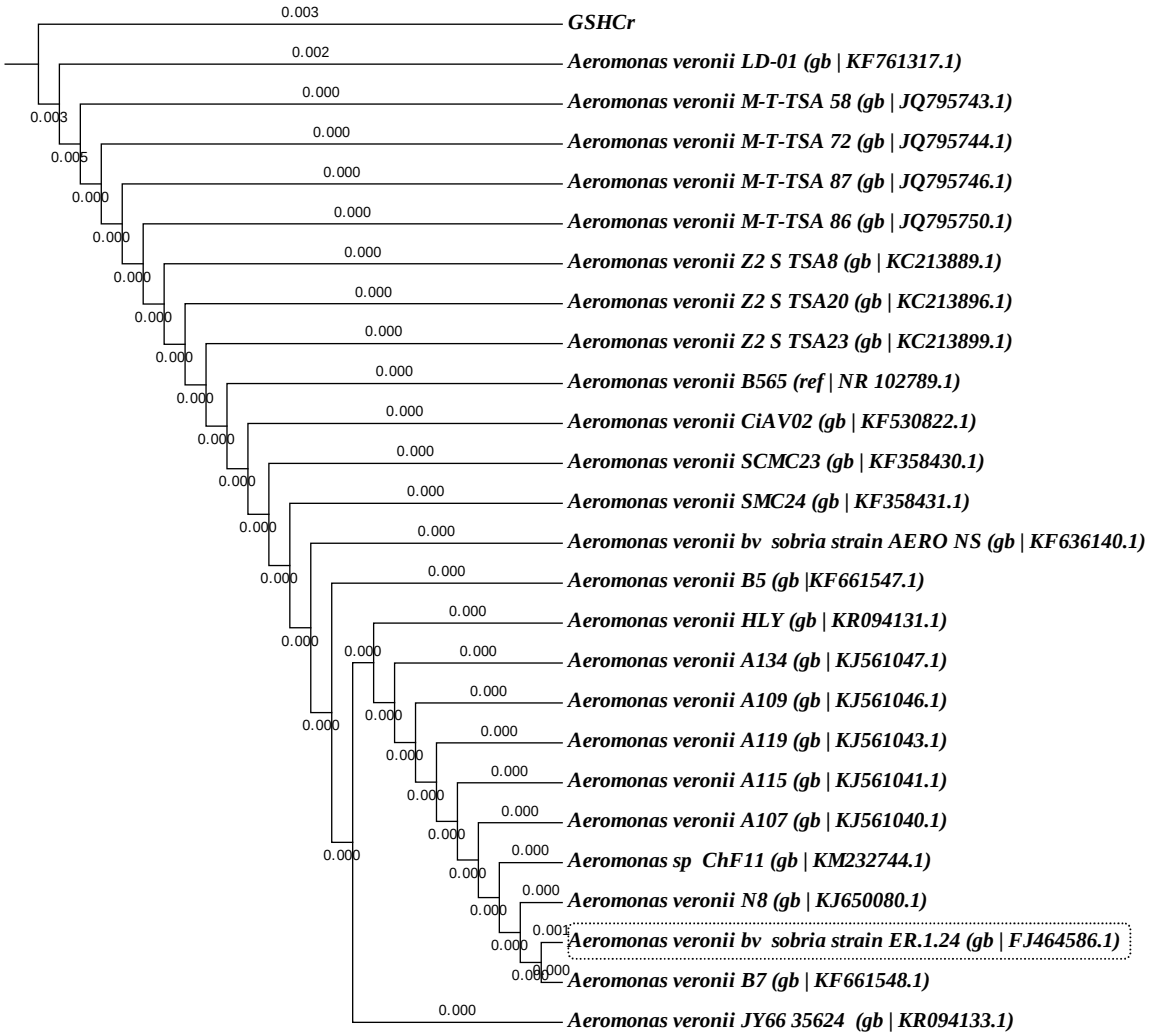


Figure 3.17 Unrooted phylogenetic tree for the isolate GSH/Cr (creamy colony forming bacteria isolated from gill, skin and head surface of *Catla* collected from Ullapara, Sirajganj) with distance with first 25 matches. Rectangular box indicates the first match.

3.9 Comparison of nucleotides among the isolates

Among the nine isolates two were identified as different strains of *Aeromonas* sp., another two were *Brevibacillus* sp. and three were *K. oxytoca*. They were different on

their nucleotide arrangement and were found similar within them as 91.13, 85.0 and 86.22% respectively. This relationship was determined by Clustal Omega (version 1.2.1) available on NCBI webpage and used for clustal matching. Nucleotide sequence obtained from chromatogram and arranged (Appendix V – XIII).

Multiple sequence alignment between two strains of *Aeromonas* sp.

```
CD       CAT---TACCGGCAGTCTACAGTGCGAGTCGGCGGTAGCGGGGAGATGTAGCTTGCTACT
GSH/Cr   TATAGGCGAGAGTCCTCACAGATGCGAGTCGGCGGCAGC-GGGGAGAGTAGCTTGCTACT
          **         *   **     ************* *** ***    *************

CD       TTTGCCGGCGAGCGCGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGGCGAGGGGGA
GSH/Cr   TTTGCCGGC-GAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGA
         *********      ********************************** *********

CD       TAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTT
GSH/Cr   TAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTT
         ************************************************************

CD       CGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTC
GSH/Cr   CGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTC
         ************************************************************

CD       ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACA
GSH/Cr   ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACA
         ************************************************************

CD       CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGA
GSH/Cr   CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGA
         ************************************************************

CD       TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAG
GSH/Cr   TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAG
         ************************************************************

CD       GAAAGGTCGGTAGCTAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTA
GSH/Cr   GAAAGGTTGGTAGCTAATAACTGCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTA
         ******* *********** ****   *********************************

CD       ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GSH/Cr   ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
         ************************************************************

CD       GTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGA
GSH/Cr   GTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGA
         ************************************************************

CD       ATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCCATGTGTAG
GSH/Cr   ATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAAT-TCCAGGTGTAG
         ********************************************** **** ******

CD       CGGTGAAATGCGTAGAGATCTGGGAGGATACCGGTGGCGAAGGCGCCCCTGGA-CAAAGA
GSH/Cr   CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGCCCCCTGGACAAAGA
         **********************  * *********************  *  ******

CD       CTGACGCTCAGTGCGAA---GCGTGGGGGAGCAAACATGATTAGAACGCTGCTGTGTGTA
GSH/Cr   CTGACGCTCAAGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGAAACGCGTGTGTGATA
         **********  * *     ***************** ********    *       **

CD       TATCACAGG
GSH/Cr   ACCAAAAG-
             * **
```

Multiple sequence alignment between two strains of *Brevibacillus* sp.

```
YD      GCTTGCGGCGCTATAATGCAGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGA
CYD     GGTGGGGCATCTATAATGCAGTCGAGCGAGTCGCTTCGGGGGCTAGCGGCGGACGGGTGA
        * * * *   ********************** ***************************

YD      GTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATAACATGGGGAAACTCATGCTAATAC
CYD     GTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATAACATGGGGAAACTCTTGCTAATAC
        ************************************************** *********

YD      CGGATAGGGTCTTCTCTCGCATGAGAGGAGACGGAAAGGTGGCGCAAGCTACCACTTACG
CYD     CGGATAGGGTCTTCTCTCGCATGAAATGAGACGGAAAGGTGGCGCAAGCTACCACTTGCT
        ************************ * ****************************** *

YD      GATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCG
CYD     GATGGGCCTGCTGCGCATTAACTAGTTGGGGGGGTAACGGCCTACCAAGGCGACGATGCC
        *********** ******** ******** ****************************

YD      TAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
CYD     TAACCCACCTGAAAGGGTGACCGGCCACACTGGGACTGACACACCGGCCACACTCCTACG
        ** ** ****** ************************** **** * *** *********

YD      GGAGGCAGCAGTAGGGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG
CYD     GGAGGCACTCCTACGGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG
        *******    ** ********************************************

YD      AACGATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGACGAACAAGTACCGTTCGA
CYD     AACGATGAACGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGACTAACAAGTACCGTTCGA
        ********* ********************************* ****************

YD      ACAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CYD     ACAGGGCGGTACCTTGACGGTACCTGACTAGAAAGCCACGGCTAACTACGTGCCAGCAAC
        **************************** ***************************** *

YD      CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CYD     CGCGGTAATACGTACGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
        ************** ********************************************

YD      CGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAACCCCGGTTCGCA--TCGGAAACT
CYD     CGGCTATGTAAATCTGGTGTTATAGCCCTGGGCTCAACCCCCGGCTGCGTATCCCAGACT
        *********** ********** ***** ************ *   **   **  * ***

YD      GTGTAGCTTGAGTGCAGAAGAGGAAAGCGGTATTCCACG---TGTAGCGG-TGAAATGCG
CYD     GTGTAACTTGAGTGCAGAAGACGTAAAGCTGCTATATCCACGTGTAGCGATTTACATGCG
        ***** *************** * **      *    *    *******  * * *****

YD      TAGAGATGTGGAGGA---CACCAGTGGC-GAAGCGGCTTTCTGGTCTGTAACTGACGCTG
CYD     TAGAGATTGTGGAGGCAACACCATTGGCGAAAGCAGCTTTCTCGTCTGTACTG-ACTCTG
        *******   *  *    ***** ****  **** ******* *******    ** ***

YD      AGGCGCGAAAGCGGTGGGGAGCAAACAGATTAGAACGTCTGCTGATGATGAGATCCAA
CYD     TATGTACTAAGCGCTGGAGGAGCAAACGATGAGATACGCCGGGGATATTCACACCCGG
                ***** *** *    **  *** ***    * *  ***  * * * **
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Multiple sequence alignment among three strains of *K. oxytoca*

```
CK      CCTTT-AG-GTCAGTCTACCGTCCGAGTCGGCGGTAGCACAGGAGTGCTTGCTCTCGGGT
WK      -CGTA-AC-GGGAGTCTACCA—TGCGGTCGACGGTAGCACAGAGAGCTTGCTCTCGGGT
SwCr.   CCTAACCGGGGCCCTAACCGTCCAAGTCGAACGGTAGCACAGGAGAGCTTGCTCTCGGGT
         *       *    *   *                *        *** **************

CK      GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
WK      GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
SwCr.   GACGAGTGGC-GGAGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
        ********** *  ********************************************

CK      TGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT
WK      TGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT
SwCr.   TGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT
        ************************************************************

CK      CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGG
WK      CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGG
SwCr.   CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGG
        ************************************************************

CK      CGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA
WK      CGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA
SwCr.   CGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA
        ************************************************************

CK      GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
WK      GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
SwCr.   GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
        ************************************************************

CK      ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCA
WK      ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGA
SwCr.   ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCG
        ************************************************************

CK      GTAAGGTTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
WK      GTAAGGTTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
SwCr.   TTGAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
         * ****************  ***************************************

CK      GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
WK      GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
SwCr.   GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
        ************************************************************

CK      GCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT
WK      GCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT
SwCr.   GCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT
        ************************************************************

CK      TCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGGTGAA
WK      TCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG-AA
SwCr.   TCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA
        *************** ************************************   **

CK      ATGCGTAGAGATCTGGAGGAATACCCGGTGGCGAATGCGCCCCC--TGACAAAGACTGAC
WK      ATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAAGGCGGCCCCCTGGACAAAGACTGAC
SwCr.   TGCGTAGAGATCTGGGAGGAATACCCGGTGGCGAAGGC-GGCCCCTGGACAAAGACTGAC
                  *********** *  *   ** **   ***   *************

CK      GCTCAGT-GCGA-AAGCGTGGGG-GAGCAAACAGATTAGA-AACTCTGTGATGATATTAC
WK      GCTCAGGTGCGA-AAGCGTGGGG-GAGCAAACAGGATAAGAAACTGTGTGG--TTGATAC
SwCr.   GCTCATGGTGCGAAAGCGTGGGGGGAGCAAACAGGATTAGATACTCTTGTTGAGTATCTC
        *****        ********** **********  *     *** *       *    *

CK      CAA-
WK      AAAA
SwCr.   AAAT
         **
```

CHAPTER 4: DISCUSSION

Disease has become one of the major limiting factors in aquaculture production of Bangladesh, especially with the recent increase of aquaculture practices in order to fulfill the protein deficiency of the rural people. Rural small-scale aquaculture is the extensive or semi-intensive low- cost farming of aquatic organisms by households or communities, using technology appropriate to their resource base (Edwards and Demaine 1997). Common diseases of freshwater fishes are ulcers including EUS, septicemia disease, tail and fin rot, gill rot, dropsy, various types of fungal, parasitic and protozoan disease (Chowdhury 1997). Ulcer types of diseases including EUS are often confusing for their expression and causative agents. These are found to occur throughout the year in both farmed and wild fishes. Due to lack of proper knowledge of fish health management, farmers have to fail in most cases to control the disease and become helpless to save their fish stock. Information on rural based small- scale farmers pond regarding disease status, their causative agents, suitable control measures etc. are very important to these farmers who contribute a lot to increase total fish production in the country (Chowdhury *et al*. 2003).

Information on the occurrence, pathogenicity and isolation of bacteria in fish cultures in Bangladesh is scarce. Most of the publications on isolation and characterization of bacterium are carried out from soil and water samples. However, bacteria isolated from fishes are characterized on the basis of biochemical properties. The lack of a specific medium for culturing pathogenic bacteria from diseased fishes is another obstacle for isolation of the bacterium. In this work we used different medium with little modification from manufacturer's instruction, which enabled the development of colonies of pathogenic bacteria as well as probable strains of other bacteria resides in water sample. A large number of replications were required to purify the colonies of the desired species.

Fish farming typically involves the enclosure of fish in a secure system under conditions in which they can thrive. Interventions in fish life cycles range from exclusion of predators and control of competitors (extensive aquaculture) to enhancement of food supply (semi-intensive) to the provision of all nutritional requirements (intensive). Intensification implies increasing the density of individuals, which requires greater use and management of inputs, greater generation of waste products and increased potential

for the spread of pathogens (Naylor *et al*. 2000). Diseases are a significant threat to the health of free-ranging and hatchery-reared populations of fishes, including a suite of important sport and restoration species. Prevention of pathogen introductions and disease transmissions is the most prudent and cost-effective means for management of fishery resources (Piper *et al*. 1982). Because fish reside in, or are reared in open-environment waters, and pathogens are transmitted horizontally via the water column, diseases are a recurring concern. In the event of a disease outbreak, quick intervention with control and treatment measures is essential to preclude or minimize mortality and disease spread to other populations. With a bacterial etiologic agent, effective disease control can be dependent upon identification of the pathogen by primary bacterial culture (Starliper 2008).

Bacterial pathogens are a major cause of infectious diseases and mortality in wild fish stocks and fish reared in confined conditions. Disease problems constitute the largest single cause of economic losses in aquaculture (Meyer 1991). Concurrent with the rapid growth and intensification of aquaculture, increased use of water bodies, pollution, globalization, and transboundary movement of aquatic fauna, the list of new pathogenic bacterial species isolated from fish has been steadily increasing (Harvell *et al*. 1999). In addition, the virulence and host range of existing pathogens has also been increasing. Poor water quality and high stocking densities often promote outbreaks of pathogens and subsequent declines in farm productivity; movement of stocks for aquaculture purposes can also increase the risk of spreading pathogens (Naylor *et al*. 2000).

There is no single technique suitable for the recovery of all known bacterial fish pathogens. Scientists need to use a combination of methods and incubation conditions to achieve pure cultures. Even so, not all taxa are culturable: for example *Candidatus* has not been cultured *in vitro* (Austin and Austin 2012).

Useful information for the diagnosis of fish disease includes:

- Gross clinical signs of disease on individual fish;
- Internal abnormalities apparent during post-mortem examination;
- Histopathological examination of diseased tissues;
- Bacteriological examination of tissues (this requires special dexterity to avoid contamination by the normal bacterial micro flora present on the surface and in the intestinal tract of fish, and in water; special contamination problems may be encountered with the examination of small fish, such as fry).

Renowned and popular phenotypic tests (*viz*. gross colony morphology, Gram staining, a set of biochemical tests, carbohydrate utilization, antibiotic susceptibility, CFU, MIC, and MBC determination, growth optimization) were performed to identify the bacterial isolates obtained from diseased carp fish collected as of various seasons and months of the year 2012 and 2013 from two different sites of Rajshahi area (Kakanhat and Darusha) and Ullapara, Sirajganj.

Most of the above-mentioned phenotypic tests have been derived from medical microbiology. Nevertheless, careful attention to detail will generate useful data about bacterial fish pathogens. Undoubtedly, more modern methods will eventually enter the realms of fish microbiology. These methods may include the development of highly reliable rapid techniques, such as offered by high-pressure liquid chromatography and mass-spectrometry. Moreover, lipid analyses could be adapted further for fisheries work. Serological techniques, such as those involving ELISA and monoclonal antibodies, are steadily entering the domain of the fish disease diagnosticians. In addition, molecular genetics techniques, notably gene probe technology, are under evaluation in several laboratories (Austin and Austin 2012).

Regarding the biochemical characterization, a wide variety of behaviours were observed among the different isolates. There has been a progressive increase in the number of new bacterial taxa associated with fish diseases and the emergence of so-called unculturables (Austin and Austin 2012).

With few exceptions, fish bacteria are inhibited by incubation temperatures above 30°C and the optimal temperatures for diseases from Aeromonads bacteria are 22°C and above (Starliper 2008) which are similar to the findings of the present work. Mainly nutrient agar media was used for the growth of the isolates. Besides that, another two routinely used media (yeast extract mannitol agar, YEMA and basal salt media, BSMY) was tested to show their characteristics.

In some reports the terms utilization and fermentation have been used interchangeably and it has been difficult to ascertain which result was reported from which test method. Fermentation should be used to describe a result from the breakdown of a carbohydrate or ‘sugar’ reaction such as used in the conventional biochemical media and the API 20E. A pH indicator in the medium is able to detect the acid change caused by the breakdown

products. Utilization should only refer to tests that determine an organism's ability to use a substance as a sole carbon source. There is usually no pH indicator in the medium and growth is observed as an increase in opacity or turbidity of the media. The exception is Simmons citrate medium, which determines an organism's ability to use citrate as a sole carbon source. There are no other nutrients in this medium. On the other hand, Christensen's citrate method contains other nutrients and is not a utilization test for citrate as a sole carbon source (Cowan and Steel 1970, MacFaddin 1980).

Problems were encountered with the reliability of some conventional biochemical tests, notably the methyl red test and Voges-Proskauer reaction, fermentation and gas production from different carbohydrates. As some of the isolates showed variable results for catalase and oxidase activity, there is a dilemma on confirming the isolates. Finally they were confirmed by 16S rRNA gene sequencing which was cost expensive as well as need more sophisticated laboratory which is not available in our country.

***Aeromonas* spp.**: The scientific literature abounds with references to aeromonads as fish pathogens, and it is often difficult to distinguish the primary pathogen from opportunist or contaminant (Austin and Austin 2012). *Aeromonas* spp. are recognized as an autochthonus inhabitants of aquatic environments (Hazen *et al*. 1978, Kaper *et al*. 1981, Larsen and Willeberg 1984). Some of them are pathogenic for poikilothermal animals, such as frogs, snakes or fish and in homeothermal animals and humans (Popoff 1984, Altwegg and Geiss 1989, Janda 1991). It has also been frequently isolated from the lesions of EUS fishes (Anonymous 1986, Llobrera and Gacutan 1987, Roberts *et al*. 1990, Subasinghe *et al*. 1990, Torres *et al*. 1990). This disease is a serious threat to the freshwater fish production of Southeast Asian countries. It causes mass mortalities both cultured and wild fish species every year. *Aeromonas* spp. contribute to the pathogenesis of the disease (Costa and Wijeyaratne 1989).

Members of the genus *Aeromonas* are ubiquitous in most types of fresh water including lake and river water, domestic sewage and raw and processed drinking water (Holmes *et al*. 1996). Some mesophilic *Aeromonas* species have been suspected of being opportunistic human pathogens, especially in association with gastrointestinal diseases including traveller's diarrhoea and paediatric gastroenteritis (Joseph 1996). In a previous study initiated by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) a set of *Aeromonas* isolates originating from humans with diarrhoea,

healthy controls and surface water were compared with regard to their virulence properties and their taxonomic identity (Kühn *et al*. 1997). Huys *et al*. (2002) described *A. hydrophila* subspecies *dhakensis* subspecies nov. isolated from children with diarrhea in Bangladesh. Martínez-Murcia *et al*. (2008) isolated *A. aquarioum* sp. nov. from aquaria of ornamental fish.

Species of *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments. Although historically the *Aeromonas* genus has been placed in the family Vibrionaceae (Popoff 1984), there have been proposals to place it in its own family, the Aeromonadaceae (Colwell *et al.* 1986). The aeromonads share many biochemical characteristics with members of the Enterobacteriaceae, from which they are primarily differentiated by being oxidase-positive. The genus includes at least 13 genospecies, among which are the mesophilic *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, and the non-motile, psychrophilic *A. salmonicida*. *A. salmonicida* is a fish pathogen and has not been associated with human infection. By contrast, the mesophilic species have been associated with a wide range of infections in humans (Janda and Abbott 1996). Although members of this genus have classically been divided into three biochemically differentiated groups (typified by *A. hydrophila*, *A. caviae*, and *A. sobria*), these contain a number of genospecies, to which new species have been added (Carnahan and Altwegg 1996). Currently the genus is made up of 17 DNA hybridization groups representing a range of genospecies and phenospecies (WHO 2002).

Aeromonas spp. have previously been isolated from EUS diseased fish in the Indo-Pakistan region by Iqbal *et al*. (1998). They found that 27% *Aeromonas* isolates from fish with EUS in Malaysia, Thailand, and Bangladesh belonged to *A. veronii* biovar sobria. In agreement with these findings, fish isolates from mucilage of *Labeo* (Darusha, Rajshahi) and gill of *Catla* (Ullapara, Sirajganj) were also genotypically identified as *Aeromonas* sp. and *A. veronii* biovar sobria respectively, indicating that this *Aeromonas* species may constitute an important causative agent of fish disease in this geographic area.

The mesophilic aeromonads have been commonly isolated from patients with gastroenteritis although their role in disease causation remains unclear. They are also associated with sepsis and wounds, and with eye, respiratory tract, and other systemic infections (Janda and Duffey 1988, Janda and Abbott 1996, Nichols 1996). Many of the

systemic infections arise following contamination of lacerations and fractures with *Aeromonas*-rich waters. The species principally associated with gastroenteritis are *A. caviae*, *A. hydrophila*, and *A. veronii* biovar sobria (Joseph 1996); *A. caviae* is particularly associated with young children (under 3 years of age). Many studies have resulted in the isolation of several species of *Aeromonas* from patients with gastroenteritis, and these have been extensively reviewed (Altwegg and Geiss 1989, Janda 1991, Joseph 1996). There has been considerable debate as to whether the mesophilic aeromonads are primary enteropathogens, prompted largely by failure to establish significant infection in volunteer studies. In a study in which 57 people were challenged using five strains of *A. hydrophila* with doses ranging from 104 to 1010 organisms, only two individuals developed diarrhoea-one had mild diarrhoea after a dose of 109 organisms and the other developed moderate diarrhoea after a dose of 107 (Morgan *et al*. 1985). The value of these data is limited, as the strains used were poorly characterized and some were not demonstrably enterotoxigenic (Gosling 1996). However, there have been reports of laboratory-acquired infections in microbiologists who (unintentionally) ingested significant doses of *Aeromonas* and developed self-limiting diarrhoea (Joseph 1996).

The genus *Aeromonas* comprises a collection of oxidase and catalase-positive, glucose-fermenting, Gram-negative, rod-shaped, generally motile bacteria (Popoff 1984). In this study, the two isolates were agreed with these descriptions of the genus *Aeromonas*. These species can be identified by routine biochemical properties such as esculin hydrolysis, gas production from glucose, lysine decarboxylation, fermentation of arabinose and salicin etc. (Janda *et al*. 1984, Bryant *et al*. 1986a, b, Kuijper *et al*. 1989). In the present study nine isolates from diseased fishes were investigated to identify the *Aeromonas* species phenotypically.

Initial recognition of *A. hydrophila* as the causal agent of haemorrhagic septicaemia was reported by several authors (Sanarelli 1891, Schäperclaus 1930, Haley *et al.* 1967), and it has been recovered as a pathogen from a wide variety of freshwater fish species, including ornamental fish (Hettiarachchi and Cheong 1994, Pathiratne *et al.* 1994) and occasionally from marine fish, e.g. ulcer disease of cod (Larsen and Jensen 1977). The aetiological agent has been considered to be the dominant cause of motile aeromonas disease in China (Nielsen *et al.* 2001), and may well have worldwide distribution.

However, some doubt has been expressed over its precise role as a fish pathogen (Heuschmann-Brunner 1965, Eurell *et al.* 1978, Michel 1981) with some workers contending that it may be merely a secondary invader of already compromised hosts. Conversely, other groups have insisted that *A. hydrophila* constitutes a primary pathogen. *A. hydrophila* has been credited with causing several distinct pathological conditions, including tail/fin rot and haemorrhagic septicaemias (Hettiarachchi and Cheong 1994). The organism may be found commonly in association with other pathogens, such as *A. salmonicida.* There is evidence that prior infestation with parasites, namely *Ichthyophthirius multifiliis* (= Ich) in channel cat fish, may lead to the development of heightened bacterial populations (*A. hydrophila)* in the gills, kidney, liver, skin and spleen and mortalities by *A. hydrophila* (Xu *et al*. 2012a,b). Haemorrhagic septicaemia (also referred to as motile aeromonas septicaemia) is characterized by the presence of surface lesions (which may lead to the sloughing-off of scales), local haemorrhages particularly in the gills and vent, ulcers, abscesses, exophthalmia and abdominal distension.

A. hydrophila found as methyl red negative and Voges-Proskauer reaction positive by Paterson (1974), Larsen and Jensen (1977), Allen *et al.* (1983a) and Popoff (1984) which did not match the findings of the present study, but production of acid from various carbohydrates resembles the same with aforesaid authors. *A. allosaccharophila* was found as not to susceptible to sulphadimethoxine or trimethoprim and acid was not produced from lactose (Martínez-Murcia *et al*. 1992) which somehow conflict with the present findings. In contrast to the usual characteristics of *A. salmonicida*, the majority of isolates of *A. hydrophila* were capable of growth at 37°C and are, indeed, motile (Allen *et al.* 1983b, Austin *et al.* 1989).

All strains of motile *Aeromonas* species are positive for the fermentation of glucose and maltose and are negative for urease and fermentation of xylose. A subspecies of *A. hydrophila*, *A. hydrophila* ssp. *dhakensis* has been found negative for acid production from L-arabinose, whereas *A. hydrophila* ssp. *hydrophila* is positive (Huys *et al*. 2002). There are also varying reports for utilization of citrate and reaction for MR. Citrate is said to be positive for 60% of strains of *A. hydrophila,* and MR is positive for 53% of strains (Abbott *et al*. 1992). *A. popoffii* type strain LMG 17541 is indole-negative, but other strains are indole-positive (Huys *et al*. 1997). In the flow

chart of Bergey's manual of determinative bacteriology *Aeromonas* has been characterized as Gram-negative rod shaped bacilli which is oxidase positive, ferment glucose and produce acid, NaCl is not required for growth. *A. hydrophila* and *A. veronii* is defined as motile and *A. salmonicida* is non-motile, where *A. hydrophila* is H_2S positive and *A. veronii* is negative.

It has been reported that the organism *Aeromonas* is rife in freshwater (Heuschmann-Brunner 1978, Allen *et al*. 1983b), aquatic plants and fish (Trust and Sparrow 1974, Ugajin 1979) and fish eggs (Hansen and Olafsen 1989), and may be associated with invertebrates, from where it will be readily available for infection of fish, although the epizootiology of *A. hydrophila* has not been considered in any great detail. The evidence points to a stress-mediated disease condition (Bullock *et al.* 1971), in which mortalities, if indeed they occur at all (Huizinga *et al.* 1979), are influenced by elevated water temperatures (Groberg *et al.* 1978, Nieto *et al.* 1985). At the time of collection of diseased fishes infected by *Aeromonas* sp. from Darusha area, water and atmospheric temperatures were recorded as 26.7°C and 32.2°C respectively. Water and air temperature were recorded as 17-18°C and 28.2°C respectively at the time of collection of infected *Catla* from Ullapara. During the collection period of samples water and atmospheric temperatures were elevated as well as humidity was in the range of 60-98% which may play a role for the increasing activity of bacterial population and infection.

***Bacillus* sp.**: The initial outbreak of disease during 1989–1991 led to mortalities of 10–15% of farmed populations of *Clarias carpis*, *C. gariepinus*, *C. nigrodigitatus*, '*Heteroclarias*' and *Heterobanchus bidorsalis* in Nigeria (Oladosu *et al.* 1994). Diseased fish were characterised by weakness, lethargy, emaciation and generalized necrotising dermatitis, with death occurring in a few days. Blood tinged fluid was present in the peritoneal cavity. Petechia and focal necrosis was evident in the liver and kidney. The spleen was enlarged, soft and friable. The myocardium was described as soft and flabby. The stomach was hyperaemic (Oladosu *et al.* 1994). Gram-positive rods of 1–4 µm in length were observed. It should be emphasized that skin lesions revealed the presence of *Aeromonas* and *F. columnare.* Bacillary necrosis was described in farmed populations of cat fish (*Pangasius hypophthalmus*) from the Mekong Delta, Vietnam. Mortalities among fish that did not otherwise respond to treatment were observed, and the disease signs centred on 1–3 mm diameter white necrotic and

granulomatous areas in the kidney, liver, spleen and viscera. Apart from the presence of mostly myxosporean parasites, an organism considered as an unspeciated *Bacillus* was recovered (Ferguson *et al.* 2001). Colony and cellular morphology as well as biochemical characteristics of *Bacillus* found to be similar to the findings of such previous study.

Oladosu *et al.* (1994) relied on nutrient agar and incubation at the comparatively high temperature of 37°C for an unspecified period to isolate *Bacillus* spp. which is similar to the present findings but the time required for the bacterial growth is 18-20 hours. Liquid nutrient consume less time (16-18 hours) than to the nutrient agar which may be due to bacterial easiness to get their nutrient for growth.

Oladosu *et al.* (1994) infected *Clarias gariepinus* via the oral and subcutaneous routes with a comparatively low dose of 0.5 ml, which contained 1.8×10^3 cells/ml. Thus, 60 and 30% mortalities were achieved over a 3-week period by oral and subcutaneous challenge, respectively. Ferguson *et al.* (2001) reported that 2×10^7 cells of the putative *Bacillus* injected intraperitoneally led to clinical disease. There has been occasional mention of *B. cereus* as a fish pathogen causing branchionecrosis in common carp (Pychynski *et al.* 1981) and striped bass (Baya *et al.* 1992).

***Brevibacillus* spp.**: Genus *Brevibacillus* has been more recently described, and now recognized to comprise a separate bacterial family, Paenibacillaceae (De Vos *et al*. 2009). *Brevibacillus* sp. is as former *Bacillus* species and associated with the soil, isolates have been found in the dairy environment, and some are used in industrial microbiology applications. There is little information about their use as probiotic agents. A strain of *B. brevis* was studied for its use for biocontrol of plant pathogens due to their antimicrobial production (Edwards and Seddon, 2001). Through extensive literature survey *Brevibacillus* did not found as pathogenic and it is rarely associated with infectious disease. Growth of *Brevibacillus* spp. occurs at pH 5.6 or 5.7 and at 50°C is variable, however optimum growth occurs at pH 7.0 and the temperature is 30°C as well as growth is inhibited by 5% NaCl (Shida *et al*. 1996). Arya and Sharma (2014) found *B. borstelensis* as Carbendazim (a fungicide used in agricultural crops) degrading agent and this bacterium can grow at 25-40°C, at pH 5.2-10.0, NaCl tolerance 2-10%. *B. borstelensis* strain 707 a thermophilic bacterium was isolated from soil and can degrade polyethylene as their sole carbon source (Hadad *et al*. 2005). Another species

B. brevis showed tolerance against a heavy metal Cd (Cadmium) and can influence nodule formation in plants (Vivas *et al*. 2005). So, this bacterium may come to fish mucilage from the soil and pond water. A phylogenetic tree based on 16S rRNA gene sequences antagonistic bacteria and type strains were established.

Klebsiella oxytoca: *Klebsiella* are renowned as opportunists, frequently resistant to antibiotics and nosocomial infections. Varieties of *Klebsiella* have been reported for sepsis, wound infections, infections of the urinary and respiratory tract in human. This is facultatively pathogenic bacteria; disease only manifests if host organism immune defenses are weakened. *K. pneumoniae* is the well known species causing pneumonia and they produce white pigmented colony (Austin and Austin 2012) which reflects the present findings. *K. pneumoniae* found as motile by flagella, fermentative, acid producer from maltose showing growth at 30°C and 37°C, and can grow without NaCl but indole and oxidase negative (Austin and Austin 2012). In the flow chart of Bergey's manual of determinative bacteriology *Klebsiella oxytoca* has been characterized as Gram-negative bacilli which is lactose fermenter, indole positive, citrate positive, VP positive and H_2S negative. Some members of the genus *Klebsiella* have been implicated in human disease or can be the cause of opportunistic infections including bacteraemia, meningitis, urinary tract infections and wound infections (Baylis *et al*. 2011), and are therefore important in clinical settings.

***Pantoea* sp.**: Hansen *et al*. (1990) reported *P. agglomerans* which is described as *Enterobacter agglomerans* as catalase producer which does not reflect the present findings. Otherwise it is almost the same in nature with all the properties. They also mentioned that growth occurs at 4-37°C but not at 40°C, and in 0-6% (w/v) but not 8% (w/v) NaCl. In Wikipedia (the free encyclopedia), it has been described that the genus *Pantoea* is "lactose fermenter" which did not support the present findings as they cannot ferment lactose of MacConkey agar media. Austin and Austin (2012) reported *P. agglomerans* as yellow pigmented colony producer, motile by flagella, fermentative, positive at methyl red test and Voges-Proskauer reaction and ability to grow at 30°C and 37°C.

Haemorrhages were also recorded in the musculature. Otherwise, there was an absence of disease signs in the internal organs (Hansen *et al.* 1990). There has been some debate over the precise taxonomic relationship of the organism, which has been classified as

E. agglomerans, *Erwinia herbicola* and now as *P. agglomerans* (Grimont and Grimont 2005). The source of the pathogen was unknown (Hansen *et al.* 1990).

The genus *Pantoea* contains seven validly-published species, which are primarily known as plant-associated or phytopathogenic bacteria, but some have also been linked with human infections. Over the past 12 years, there have been increasing isolations of *Pantoea* strains from a range of hosts and locations which cannot be conclusively identified. This has highlighted the need for a rapid, molecular-based technique to conclusively characterize these strains.

P. agglomerans has been focused upon as a useful Gram-negative bacterium of edible plant origin. *P. agglomerans*, which can fix nitrogen and solubilize inorganic phosphorus, has been isolated as a symbiotic bacterium not only from wheat (Lindh *et al*. 1991) but also from rice (Feng *et al*. 2006) and sweet potato (Asis and Adachi 2004) and has been investigated for its growth promoting activity in these plants (Asis and Adachi 2004, Feng *et al*. 2006).

Members of the genus *Pantoea* are Gram-negative, motile rods belonging to the family *Enterobacteriaceae* and display ecological versatility, as they are commonly recovered from water, soil, insects, and plants (Rezzonico *et al*. 2009, Smits *et al*. 2011). *P. ananatis* is associated primarily with plants, as epiphyte or endophyte, and as an emerging phytopathogen infecting a wide range of important crop and forest plants (e.g., maize, onion, rice, pineapple, *Eucalyptus*) (Coutinho and Venter 2009). *P. ananatis* has also been reported as an occasional clinical isolate and a presumptive opportunistic human pathogen, associated with septicemia following penetrating trauma with plant material, nosocomial infections due to exposure to contaminated hospital materials, and secondary complications of preexisting illnesses (De Baere *et al*. 2004, Rezzonico *et al*. 2009, 2012). The genus *Pantoea* among others, found by Rezzonico *et al*. (2009) that they entertain bivalent interactions with both plants and human tissues.

Genomic evolution and adaptive strategies of bacterial fish pathogens are poorly understood and lags far behind that of human and terrestrial animal pathogens. A detailed knowledge of the genome sequences of bacterial fish pathogens and how the genomes of the pathogenic species or strains evolved from nonpathogenic ancestors or counterparts will help us better understand their pathogenicity mechanisms and

strategies of host adaptations. This information will help identifying novel vaccine and drug targets in the genomes of pathogens (Sudheesh *et al*. 2012).

There is evidence that molecular techniques have been used with increasing regularity for bacterial pathogens. A timely overview of PCR with emphasis on validation of the techniques and problems relating to diagnosis has been published (Hiney and Smith 1998). An ideal situation would involve techniques that could recognise and differentiate between multiple diseases, and this has been achieved with multiplex PCR. Del Cerro *et al*. (2002) detected simultaneously *A. salmonicida, F. psychrophilum* and *Y. ruckeri* in fish tissues, recognising the equivalent of 6, 0.6 and 27 CFU, respectively. Similarly, González *et al*. (2004) used a multiplex PCR and DNA microarray, and achieved the simultaneous and differential diagnosis of *A. salmonicida, Photobacterium damselae* subsp. *damselae, Vibrio anguillarum, V. parahaemolyticus* and *V. vulnificus*. DNA microarrays have been used to detect fish pathogens, including *A. hydrophila*, *Nocardia seriolae*, *Streptococcus iniae, V. alginolyticus, V. anguillarum* and *V. harveyi*, and demonstrated congruence with other methods, i.e. culturing and 16S rRNA gene sequencing (Shi *et al*. 2012).

Among the molecular techniques PCR is undoubtedly the most efficient tool for the diagnosis of bacteria. Several protocols were applied, but due to the lack of data on the genome sequence of *Aeromonas, Bacillus, Brevibacillus, K. oxytoca* and *Pantoea* from water and host fish. The use of specific primers for conserved regions of bacterial DNA is the most accessible technique (Bader *et al*. 2003, Darwish *et al*. 2004).

Amplification of the 16S rDNA gene region is a simple technique to be performed for the characterization of large numbers of isolates. Data from this study show the existing genetic variation within a supposedly conserved gene, 16S rDNA, and they can explain the difficulty of establishing diagnostic protocols for molecular characterization of the studied strains. Analysis of the phylogram and its ramifications also suggests the existence of subspecies of several isolates. Dependence of the molecular technique on certain regions of the gene makes comparisons between isolates from different studies a challenge unless similar methods are applied in the analysis (Darwish and Ismaiel 2005).

Historically, scientists have seemed loath to make rapid diagnoses, preferring to adopt laborious testing regimes. There have been dramatic improvements in diagnostic improvements, encompassing recent developments in molecular biology (Austin and Austin 2012). Diagnosis has often appeared to be as much art as science, with a multitude of preferred methods adorning the notebooks of most diagnosticians (Austin and Austin 2012). However, the rapid acceptance of molecular-based methods has revolutionised diagnostics in terms of accuracy.

Identification of bacteria in most microbiological laboratories of the third world depends on their phenotypic properties because of the unavailability of molecular techniques due to financial and technical support.

A false or weak positive and negative reaction, which often occurs in routine laboratories, leads a misidentification of bacterial species. The phenotypic properties presented herein, might be a useful phenotypic scheme to identify the bacteria from affected fish in the microbiological laboratories. However, it needs 16S rDNA sequencing experiment for further identification of the phenotypic species. These experiments will help to know the relationship between the phenotypic and genotypic identification as well as the phylogenetic position of the identified isolates. 16S rRNA sequencing has helped the description of fish pathogens where phenotypic characterization alone does not permit their incorporation in classifications (Austin 2011).

The identification of bacterial species in most bacteriological laboratories is based on phenotypic properties. Although this method has been quite successful, it has not been precise enough to distinguish closely related species. Bacterial genus, with wide phenotypic variations, the problem becomes aggravated resulting in confusion of the identification, which hampered its clinical significance as well as epidemiologic aspects. In view of these, there need new methods which give best reflection of the genetic make-up of organism that would be useful for identification.

It would seem that phenotypic identification based on routine procedures in most laboratories may not be sufficient to identify bacteria to the species level. Several authors stress the necessity of using molecular methods in addition to biochemical markers for the correct identification of strains with genomic species (Altwegg 1993, Carnahan 1993). The first application of this technique to the psychrophilic aeromonads

by Popoff *et al*. (1981) demonstrated that the phenotypically circumscribed *Aeromonas* species, eg. *A. hydrophila,* comprised several genotypically different groups with genetic distances supporting species delineation.

In this study, bacteria were first identified comparing the biochemical properties, then the phenotypic identification was confirmed by genotypic identification. It appears from our study presented herein that there are not sufficient phenotypic properties, which can identify bacterial isolates with confidence.

Identification of bacterial isolates to the species level based on the phenotypic properties is imprecise and strains that possess biochemical properties atypical for a given genospecies are common. Such strains cannot be identified accurately using a limited number of phenotypic properties. Kuijper *et al*. (1989), Hickman-Brenner *et al*. (1987) and Allen *et al*. (1983b) reported that phenotypic identification of *Aeromonas* species using various published biochemical schemes or the diagnostic keys in Bergey's manual was substantially inadequate in terms of the genetic heterogenicity of most *Aeromonas* species. The authors also found the differentiation between phenotypic and genotypic identification of *Aeromonas* sp.

Aquaculture is a fast-growing food production sector and the need for antimicrobial agents varies markedly between countries. Intensification of aquaculture has led to the promotion of conditions that favour the use of a wide range of chemicals, including antibiotics, pesticides, hormones, anaesthetics and various pigments. The quantities used and the usage patterns also vary between countries and individual aquaculture operations. However, the market for aquaculture antimicrobials is small and the approval process for new compounds is expensive. Antibiotics can be used in fish to treat bacterial diseases effectively, but are not for growth promotion as in other food-producing industries. Antifoulants that contain biocides and pesticides used for treatment of certain parasites are also used in aquaculture operations. The provision of increased selection pressure leading to antibiotic resistance can occur by overuse or misuse in human medicine, livestock, agriculture, horticulture and aquaculture. There are virtually no antimicrobial agents available for treatment of molluscan or crustacean diseases and alternative control measures are therefore required. Alternatives to the use of antimicrobial agents include good husbandry, adequate feed composition, vaccines, biological control and movement restrictions through legislation. Further research is

required in areas such as vaccine development, immunostimulants and the use of probiotics (Rodgers and Furones 2009).

The use of antibiotics to control fish diseases has met with limited success and has the potential danger of antibiotic resistance development in aquatic bacteria (World Health Organization antimicrobial resistance fact sheet 194, http://www.who.int/inf-fs/en/fact194.html) (Subasinghe 1997). As aquaculture is one of the fastest growing food production industries in the world, demand for sustainable ways of combating fish diseases is gaining significance. There is tremendous scope for developing novel vaccines and therapeutic drugs against bacterial fish pathogens (Sudheesh *et al*. 2012). In a fish farm where a symptom of disease (red spot) was primarily seen, the grower applied antibiotic namely Doxy-Oxy (combination of doxycycline and oxytetracycline) with air dried supplementary feed on a regular basis of a week.

According to the American Society of Microbiology Task Force on Antibiotic Resistance (ASM 1994) "the increasing problems associated with infectious diseases in fish, the limited number of drugs available for treatment and prevention of these diseases, and the rapid increase in resistance to these antibiotics represent major challenges for this source of food production worldwide".

A number of recent reports, press releases and on-going investigations have raised legitimate public concerns about the safety of antibiotic drug usage in aquaculture (Alderman and Hastings 1998, Goldburg *et al*. 2001). Establishing the exact level of drug use and potential dangers is difficult due to lack of data, fragmented laws, regulations, jurisdictions and interpretations of reporting guidelines (OTA 1995) and large quantities of aquaculture imports from countries where legal and illegal drug use may escape documentation.

However, it is important to emphasize, that there is not a direct linkage between pounds used in aquaculture versus other agricultural uses versus human use and the risk of adding to the pool of resistance bacteria threatening public health. Uses of antibiotics in aquaculture occur in environments with vastly different pathogens, bacteria, and antibiotic selection pressures (Goldburg *et al*. 2001). Aquaculture is also associated with novel routes for people to become exposed to resistant bacteria, or genes conferring resistance to a given antibiotic with roots in aquaculture. For this reason, aquaculture

may be, or may come to contribute to the pool of antibiotic resistant bacteria triggering infections in humans disproportional to aquaculture's share of antibiotic use. Given the global nature of the antibiotic resistance crisis, any and all uses of antibiotics that are contributing to the problem should be examined.

Economic impact of diseases and biosecurity measures: The economic values of fish and fisheries species at different levels are relative to (i) farmgate level, (ii) national export level, (iii) regional and national socio-economic considerations, and (iv) stock market level. In 2006, global farmgate value of aquatic animals was estimated to USD 78.8 billion and the Global loss from diseases estimates may have different assumptions and total loss of USD 3 billion per year (1997/2001/2006) (World Bank report 2006).

Examples for economic losses due to disease

- Losses due to shrimp diseases: USD 3 billion for 11 countries (Asian + South/North America) for the period 1987-94;
- Bangladesh; 15% reduction in production (questionnaire 2004);
- In Ecuador (1999/2000), white spot disease (WSD) caused production losses valued at USD 600 – 1000 million – all production units affected, several 100,000s jobs lost in the sector;
- In China (1993) and Thailand (1996), white spot disease (WSD) alone caused more than USD 400 and 500 million in production loss;
- Annual losses finfish species in China > USD 120 million (1990 – 1992);
- Sea-lice mortalities have cost the EU salmon farming industry an estimated €14 million per year;
- Infectious salmom anaemia cost the industries of Norway, Scotland, and Canada in the order of USD 60 million (MacAlister Elliot and Partners, 1999);
- ISA outbreak in Scotland 1998/1999: £20 million;
- Annual ISA cost in Norway and Canada: USD 10-15 million (Hastings *et al*. 1999).

Cost of disease: This could be measured by adding loss (reduction in benefits) and expenditures (additional input). Expenditures includes farm level (e.g. biosecurity measures (including vaccination), treatment and extraordinary costs), national level (legislation and control and surveillance), indirect cost (cost to society (social, welfare, environmental), negative externalities and insurance/potential government

compensation) and other effects (adjustments in market share, increasing price due to lower supply). However, such cost levels are not monitored or recorded neither by the farmers nor by the fisheries sector of Bangladesh.

Impact of diseases: Productivity of healthy fish, trade of fish, environment, welfare of the aquatic organisms and human welfare are the main constraint.

Effects on productivity: Biological and economic feed conversion rate (feed intake i.e. altered ability of intake and digest the feed by fish), growth rate is affected, which may ultimately resulted in mortality or morbidity (biomass for sale is reduced and yield consumable is also decreased), flexibility in production and immunosuppression (increased sensitivity to other diseases).

Effects on trade: Disease affected trade by lower marketing margins, reduced quality and thus value of the products, negative publicity may cause reduced demand for the fish, trade restrictions (national and international) often caused in shrimp export, and loss of market access/market shares.

Environmental impact: Increasing infectious load in the aquatic environment thus lead to the introduction of new agents affecting new host species.

Welfare: Disease relates to human welfare (loss of earnings/jobs, recreation, the apprehension of a healthy environment, eating food of (potentially) sick fish, working with diseased fish) hence it is needed to focus on this event.

Biosecurity ("Prevention is better than cure"): It is defined as the process and objective of managing biological risks associated with food and agriculture (food production) in a holistic manner (FAO). The probability of avoiding introduction or reintroduction of an infectious agent to the farm (Stott *et al*. 2003) is the first step towards this. It must be based on risk profile of actual disease(s), epidemiology of the disease, efficient farm level strategies *viz.* (good management practice, testing of water parameters, quarantine of spawns and juveniles before introducing in the farm pond, and vaccination (whenever needed). Biosecurity includes legislation and on-going surveillance and control programmes and it is non-agent/disease specific.

Biosecurity activity: This will reduce the probability of infectious exposure (known and unknown) and curtail its effect (holistic), it is an additional input to the production function, it may increases output or lower the need for input, and it should be balanced between benefit and cost.

Optimization of benefits from biosecurity measures: If biosecurity measures taken in right manner there should be little risk of introduction of pathogens (risk profile of various diseases, endemic vs new disease, probability of biosecurity breakdown), there is an idea about epidemiology of the disease, thought should be given about farm characteristics, awareness of opportunity costs and it reduces the risk and increases the benefits.

The variations in the literature for different strains are also recorded, so it is needed to be aware of the difficulties encountered when identifying particular species. Hopefully this system should not prove too tedious for the workers; it is intended to give the users more confidence in naming an organism and to alert them to potential difficulties. In some cases, species are relatively newly described and different researchers have obtained different biochemical reactions. It must be stressed that difficulties arise when assessing results of some phenotypic tests when two different versions of a biochemical test have been used, where differences were noted from different manuals, and journal articles. Phenotypic tests are still the primary identification system, and therefore we must be aware of the difficulties encountered with some tests for particular organisms. Some species have been newly described on one strain only and as more information is gathered about this species it may become evident that the type strain is not in actual fact representative of the species (Janda and Abbott 2002). Therefore subsequent strains isolated and identified as belonging to this species may show slightly different results in the biochemical tests. In this case, the atypical *A. salmonicida* group different biochemical results are reported from strains isolated from different fish species. For the diagnostic laboratory trying to identify an unknown isolate, it may be difficult to say for certain that the isolate is an atypical *A. salmonicida*. However, with the phenotypic details listed of all isolates from different fish species, it may enable a more definitive identification, or at least an identification can be made with slightly more confidence. There are limitations to all phenotypic (and genotypic) identification systems (Janda and Abbott 2002).

Bangladesh is one of the 15 leading aquaculture producing countries in the world achieving a rank of 6th position (FAO 2011). Aquaculture contributed about 52.92% of total fish production of the country during 2011-12 (DoF 2013). In spite of tremendous potential production, rural freshwater aquaculture of Bangladesh has been suffering from outbreak of diseases. The common fish diseases in Bangladesh are ulcer type disease including EUS, tail and fin rot, bacterial gill rot, dropsy, fungal diseases and parasitic diseases (Chowdhury 1993). Infectious diseases caused by bacterial and fungal pathogens have been recognized as a serious threat to aquaculture in Bangladesh (Zahura *et al.* 2004). Hossain *et al.* (2011) estimated the losses due to mortality and retardation/cessation of growth of fish in ponds in West Bengal as a result of epidemic infections. In China, it was estimated that around 10% culture area is suffering from disease, with annual losses of fish production is around 15% (Wei 2002). In Bangladesh only a few studies have been carried out on the economic impact of fish diseases. Bagum *et al*. (2013) reported the average prevalence of fish diseases was highest (27.0%) in Rajshahi followed by Mymensingh (24.6%) and Sylhet (18.3%) districts. About 76.70% diseases were reported mainly in winter season, 7.70% in summer, 7.33% in both the seasons, 6.70% in rainy season and 1.70% in any season in ponds, and highest number of farmers (27.33%) used lime and salt to treat fishes, and the highest average economic loss was estimated as BDT 30,023 ha^{-1} yr^{-1} (14.4%) in Rajshahi.

In Rajshahi districts, maximum fish ponds do not have permanent source of water and such ponds depend for water on rainfall, rice field, and near river/ditch. Dahal *et al.* (2008) indicated that ponds receiving water from rice field and river/ditch had showed high relative risk of EUS.

In the studied ponds, fishes were infected with red spot disease and the clinical signs of the disease were red spot or haemorrhages on skin and at the base of fins, sometimes ulcers in the skin, finally lost scales and died. Red spot disease is caused by a group of motile aeromonas septicaemia including *A. hydrophila, A. sobria,* and *A. caviae*. This disease often occurs during the change from the dry to rainy season and during the flood season (Khoi *et al*. 2008). Conditions that favor red spot disease are also high stocking densities, environmental pollutants and a large amount of organic mud in the pond (Roberts 1993).

In pond of Kakanhat, Rajshahi area diseased fishes were found in winter season. Faruk *et al.* (2004) reported that about 79% disease was found in winter season. Hossain and Paul (1993) reported that the immune system of fish suppressed this time due to low temperature and fish become more susceptible to fish disease.

The pond water of Darusha area was heavily engage with chemicals because it was surrounded by paddy field where run out water entered easily at rainy season (June-August) as it was lying down from the surrounding field. No doubt this is one of the major causes of disease outbreak.

Fish farmer of Ullapra, Sirajganj area reported that gill rot was found frequently in major carps. This disease was characterized by the white margins on the fins and putrefaction of the gill or fins. It was caused by the bacteria called *Myxobacter* and *Flavobacterium* (Turnbull 1993). This disease occurs mostly among the young ones during summer months. Besides the high temperature, humid condition, high organic material settled at the bottom of the pond was also a predisposing condition for the outbreak of this disease which was noted as managerial problem.

Fish diseases are one of the major constraints resulting from intensification of aquaculture and may eventually become a limiting factor to the economics of a successful and sustainable aquaculture industry. The most commonly reported diseases was EUS, followed by red spot, tail and fin rot, grayish white spot in *H. fossilis* and dropsy were reported by Mazid (2001), DoF (2002), Faruk *et al*. (2012).

Faruk *et al.* (2012) reported that mostly lime was applied in treating brood and health management. Sultana (2004) also observed that lime is very effective and widely used common chemical in Bangladesh.

Economic losses from diseases are likely to increase as aquaculture expands and intensifies. The estimated economic loss due to fish disease varied from farm to farm based on the intensity of infection and the management practices adopted by farmers. The average overall economic loss and control cost due to disease was found BDT 24,870 ha^{-1} yr^{-1} and BDT 3,460 ha^{-1} yr^{-1}, respectively (Bagum *et al*. 2013). However, about 12.9% of the production value was lost due to fish disease. Faruk *et al*. (2004) reported that average loss due to fish disease was BDT 20,615 ha^{-1} yr^{-1}. The economic

loss due to disease could be attributed to reduced growth rate, which was possibly due to poor food conversion ratio (FCR) in infected fish. It was also observed that farmers spent extra feed to regain better FCR and ultimately better growth. Bagum *et al*. (2013) reported that the economic loss due to incidence of fish disease in Rajshahi district was the highest. This might be for higher stocking density, poor water quality, poor knowledge about fish disease control and inexperience for better fish health management techniques.

Biosecurity measures are essential in any biological production chain to control the devastating effects of diseases and maintain global trade. Cost of biosecurity is a necessary input in the production function. Models for promoting and optimizing benefits of biosecurity measures are needed to secure the understanding of the biosecurity and cost relationship so that the best practice is understood and adopted. Animal health economics and disease impact is a multidisciplinary complex issue where large set of production, epidemiological and biosecurity data required (data may be difficult to achieve).

There are some fish diseases and infections that can be transmitted from fish, and the water in which they are cultured, to humans. Although the infection of humans with fish pathogens is a relatively unusual event, it is a health risk that needs to be recognized by fish farmers and other people who handle and/or consume farmed fish. The incidence of transmission of disease from fish to humans is dependent upon several factors including the type of organism (viral, parasitic or bacterial), the susceptibility of the host (immuno-compromised individuals, presence of open wounds) and environmental factors (quality of the water, depth of penetration of fish spines). Optimum farm design, appropriate husbandry and handling, water quality management and regular fish health monitoring will reduce the risk of disease transfer from fish and their environs to workers in the aquaculture industry.

CHAPTER 5: SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

The present study was carried out from March 2012 to February 2015 to isolate and characterize the bacteria which were responsible for disease in farmed carp fishes. Sample collection was the first step for the study and samples were collected from two sites of Rajshahi and one site of Ullapara upazilla, Sirajganj district. From these three ponds this was the first report of disease outbreak. Moribund diseased fishes were collected and immediately brought to the Genetics and Molecular Biology Laboratory, Department of Zoology, University of Rajshahi along with pond water. Collected diseased fishes were *P. sarana*, *L. rohita*, *L. bata*, *C. catla*, *C. mrigala* and *H. molitrix*. From the fishes and water samples from Ullapara a total of nine (9) isolates were primarily differentiated on the basis of their colony size and colour; then sub-cultured on regular basis on nutrient agar and nutrient broth to get pure cultures for further tests.

Morphological, physiological and biochemical characteristics were detected through Gram-staining, KOH stringing, lactose fermentation, citrate utilization, catalase, cytochrome-oxidase, oxidative-fermentative, growth on triple sugar iron agar, sulfur reduction, indole production, motility investigation, urea hydrolysis, methyl red and Voges-Proskauer reaction, carbohydrate utilization, and antibiotic susceptibility test. Colony forming unit for each of the isolates were calculated on nutrient agar plate; minimum inhibitory and minimum bactericidal concentration were detected using two antibiotics ampicillin and tetracycline, and growth optimization of the isolates have been done at temperatures from 25 to 37°C, at pH from 6 to 8 and at salinity 1 to 3%.

Genomic DNA was extracted from each of nine isolates using TIANamp Bacteria DNA kit (Tiangen, China) for molecular identification through 16S rRNA gene sequence. PCR was done with extracted genomic DNA using several sets of forward and reverse primers, PCR products were run through 1.0% agarose gel containing ethidium bromide, and visualized under UV-transilluminator. Amplicons were purified using TIANquick Midi purification kit (Tiangen, China) and purity and concentration were measured using spectrophotometer Nanodrop 2000. With the obtained sequence from ABI Prism

genetic analyzer NCBI nucleotide BLAST search tool was used to find the possible matches deposited on databases. Bacterial identity was found according to the first match and FASTA sequences were downloaded and phylogenetic trees were constructed. Trees of the isolates were produced using BLAST pair wise alignments and neighbor joining tree method was used. Unrooted tree was downloaded as Newick format and trees were edited with MEGA version 6.0 software.

Semi-intensive mixed carp culture system was followed by the producers of pond of Kakanhat and Darusha area, Rajshahi and monoculture of *Catla* was practiced by the grower of Ullapara, Sirajganj from where the diseased fishes were sampled. On the spot water temperature, pH and salinity were recorded as 19.5°C, 7.9 and 0 ppt respectively for pond of Kakanhat (December 2012); 26.7°C, 8.2 and 1 ppt for the pond of Darusha area (July 2013) and for the pond of Ullapara, Sirajganj they were recorded as 17-18°C, 7.8-8.2 and 2-3 ppt respectively (August 2013).

The isolates designated as CK, WK, YK for Kakanhat; CD, YD and CYD for Darusha and SW/Cr, SW/Yel and GSH/Cr for Ullapara, Sirajganj. Colony sizes were large for all the isolates except YK, and shapes were circular while SW/Yel produced irregular colony. Isolates CK, WK, YK, CD, SW/Cr and GSH/Cr were Gram-negative rods and three isolates YD, CYD and SW/Yel were Gram-positive. Gram-negative six isolates can utilize citrate of Simmons citrate agar; isolates CK and WK can ferment lactose of MacConkey agar while other four could not. Isolates CK, WK, SW/Cr and SW/Yel were found as catalase positive while YD and CYD were weak positive on catalase activity. YK, CD and GSH/Cr did not show any activity on H_2O_2. All the isolates were found as fermentative type of bacteria. Three isolates (CD, CYD and GSH/Cr) were oxidase positive. None of the isolates reduce sulphur, five (CK, WK, CD, SW/Cr and GSH/Cr) produced indole, and three (CD, SW/Cr and GSH/Cr) were motile on motility indole urea (MIU) media. Six isolates (CK, WK, YK, CD, SW/Cr and GSH/Cr) showed positive results for methyl red test while four (YK, CYD, SW/Cr and SW/Yel) of them were Voges-Proskauer positive. Sucrose was utilized by all the isolates where SW/Yel only could ferment sucrose. Among the nine isolates six showed resistance against bacitracin. All the isolates were more or less sensitive to gentamicin, neomycin,

cephradine, doxycycline, tetracycline, ceftriaxone, ciprofloxacin, pefloxacin, mecillinam and nitrofurantoin.

The CFU/ml were calculated as 42-50×10^{10}, 39-48×10^{10}, and 37-43×10^{10} for the three isolates CK, WK and SW/Cr respectively. For the two isolates CD and GSH/Cr CFU/ml were 34-41×10^{10} and 32-39×10^{10} respectively. CFU/ml was recorded as 65-71×10^{10} and 58-64×10^{10} respectively for the two isolates YD and CYD. Isolate YK and SW/Yel formed 23-27×10^{10} and 11-16×10^{8} CFU/ml respectively. Two isolates (CD and GSH/Cr) were found ampicillin resistant, MIC for this antibiotic ranged from 320-640 µg/ml and MBC was in the range of 320-1280 µg/ml for the rest of the isolates. The MIC and MBC were found to range from 320-640 µg/ml and 320-1280 µg/ml respectively for the antibiotic tetracycline as all the isolates were sensitive to this antibiotic. Isolates showed their optimum growth in the range of temperature 28–30°C, pH 6.8–7.5 and salinity at 1%.

Five different primers were used and the combination of 8F–806R and 8F–1492R gave the more contrast single band in between 700–800 bp in comparison to 1kb plus DNA ladder. PCR was carried out with an increased volume (50 µl) using 8F–806R. PCR products were purified and prepared for sequencing PCR. The BLAST search results for the partial sequences of the 16S rRNA gene of the isolates revealed that three isolates CK, WK and SW/Cr were from the same species *K. oxytoca* but of different strains. The third isolate YK found as *Pantoea* sp.; fourth and ninth isolates CD and GSH/Cr were different strains of *Aeromonas*. The fifth and sixth isolates (YD and CYD) having almost same physiological and biochemical characteristics found as different strains of the genus *Brevibacillus borstelensis*. The isolate SW/Yel was the only member from the genus *Bacillus*.

5.2 Conclusion

Outbreaks of bacterial diseases are influenced by the susceptibility of the host, virulence of the pathogens, and quality of the environment (Wedemeyer *et al*. 1976). Therefore, control of diseases of fish and shellfish is primarily a managerial problem. It is very important for aquacultural managers to have a thorough understanding of biology, physiology, microbiology, immunology, ecology, and therapy. It is necessary for managers to be properly trained and capable of making proper evaluations of the disease problem. Reduction of losses caused by bacterial and other diseases of fishes will require integration and evaluation of all that is known about the nature of the disease, and proper and timely application of control measures (Snieszko 1978).

Assessing the impacts of disease in aquaculture system is not easy, as only acute losses are recognized and quantified. Chronic mortalities and poor growth caused by disease are generally not recognized. In order to quantify disease losses, farmers should be able to identify disease as the reason for crop loss, slow growth or poor harvest. Rural farmers are mostly resource poor with little or no knowledge of health management and have inadequate opportunities to improve management skills. Therefore, it is important to train farmers to carry out field-level diagnosis and assess the likely impacts of diseases.

A number of chemotherapeutics were reported for controlling fish disease. Liming and salting together was the most common practice followed by application of lime only, salt only, potassium permanganate, antibiotics, pesticides and insecticides. Lime is very effective in maintaining pH, water color, turbidity, increase the rate of decomposition and to treat disease fishes. Changes of environmental factors due to global warming are directly affecting the life of aquatic animals. As a result the aquatic animals are increasingly in under pressure to change their normal behaviour and become more susceptible to environmental factors.

Among the identified isolates *Aeromonas* is well known as disease causing agent in fish and also cause disease in human. Scarce information is about *Bacillus* and *Pantoea* as fish pathogen, different species of *Klebsiella* are renowned for their pathogenicity in different organisms including man. *Brevibacillus* sp. is first time isolated and identified from diseased fish. Therefore, *Bacillus*, *Pantoea* and *Brevibacillus* cataloged in the present study will give clues at upcoming days.

Research on this aspect has not yet been done systematically in the country. Considering the importance, the objective of the present study was to investigate ulcer type of diseases in the small-scale rural farmer’s pond.

5.3 Recommendations

Water source is one of the major factors for pre-stocking pond management which has a direct relationship with disease occurrence. In another partial study with the present work, it was found that drainage sewage water from homestead and domestic use burdened with *Staphylococcus* which is another cause for disease outbreak. So keeping in mind this sort of matter, entrance of water from such sources should be maintained.

Secondly, fish fry should be collected from renowned sources where all parameters (*viz.* matured and healthy brood fishes, hygienic condition of hatchery etc.) were maintained strictly. Transportation of fry and fry releasing provision has a great influence on fish health. Therefore, proper administration should be taken to minimize the chance of disease occurring factors.

Finally, diagnosis of fishes on naked eye and differentiation of diseased fishes from healthy ones is another point to restrict the havoc of disease expansion. So, awareness build up among fish producers is needed where there is a little chance to trained skilled manpower on fisheries sector.

Low cost and less time consuming culture independent techniques should be developed to identify the etiologic agents because culture dependent techniques took more time. Proper administrations on disease management need to be advocated and farmers' and manpower who are engaged with fish trade should be informed that proper steps should be taken at the time fish handling.

Though there is no evidence of *Brevibacillus* as pathogen and there is always a chance to show pathogenicity over a population where they found. So, it is needed for all the isolates especially *Brevibacillus* to test their pathogenicity on fishes through artificial challenge tests through intra-muscular of intra-peritoneal injection and immersion or bath. Study would be carried out for histological changes after artificial challenge test.

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Appendix table I Chronology of global EUS outbreak (Kamilya and Baruah 2014).

Country	Year of occurrence	Description	References
Japan	1971	First report of EUS like condition in farmed ayu (*Plecoglossus altivelis*) in Oita Prefecture	Egusa and Masuda (1971)
Australia	1972	Outbreaks of a cutaneous ulcerative condition (called red spot disease) in estuarine fish, particularly grey mullet (*Mugil cephalus*) in Queensland, Australia	McKenzie and Hall (1976)
Papua New Guinea	1975–1976	An ulcerative disease outbreak, occurred in the rivers of southern Papua New Guinea	Haines (1983)
Malaysia	1980	Typical EUS like outbreaks occurred in rice-field fishes in northern Malaysia	Jothy (1981)
USA	1980	Estuarine fish along the east coast of the United States have experienced seasonal epidemics of deep skin ulcers known as ulcerative mycosis	Hargis (1985), Dykstra *et al.* (1986)
Thailand	1981	Outbreak of EUS have occurred annually in Thailand since 1981	Ulcerative Fish Disease Committee (1983)
China	1982	Ulcerated *Labeo rohita* were first observed at the Pearl River Fisheries Institute in Guangzhou, South China	Lian (1990)
Vietnam	1983	The probable first occurrence of EUS in snakeheads came from the Mekong delta	Xuan (1990)
Lao PDR	1983	Major EUS outbreaks	Lilley *et al*. (1992)
Myanmar and Cambodia	1984	Major EUS outbreaks	Lilley *et al*. (1992)
Philippines	1985	A serious outbreak of EUS occurred at Laguna de Bay	Llobrera and Gacutan (1987)
Sri Lanka	1987	A major outbreak of epizootics occurred in freshwater and estuarine fish in western Sri Lanka	Costa and Wijeyaratne (1989)
Bangladesh	1988	Ulceration was observed in many wild species, as well as cultured Indian major carps during February 1988	Roberts *et al*. (1989)
Hong Kong	1988	Seasonal mortalities of snakeheads first started to occur in late summer 1988	Wilson and Lo (1992)

Appendix table I contd.

Country	Year of occurrence	Description	References
India	1988	The north eastern Indian states were the first to report losses in May 1988	Zoological Society of Assam (1988)
Bhutan and Nepal	1989	EUS had spread to Himalayan valley regions	Phillips (1989)
Pakistan	1996	EUS was confirmed in snakeheads from Punjab Province	DFID (1998)
Singapore	1997	Mycotic granulomatosis was found in ornamental fishes imported from Singapore	Hanjavanit *et al*. (1997)
Botswana	2006	For the first time in Africa, the wild fish stock of Zambia experienced EUS in the Zambezi–Chobe River in Botswana	Andrew *et al*. (2008)
Zambia	2007	The EUS outbreaks occurred in Zambia which share the border or connect to the Chobe–Zambezi River system	FAO (2009)
Namibia	2007	The EUS outbreaks occurred in Namibia which share the border or connect to the Chobe–Zambezi River system	FAO (2009)
Iraq	2007/2008	Suspicion reported to the OIE in 2007 and 2008, but unconfirmed. Information is available on World Animal Health Information Database (WAHID) website	EFSA Panel on Animal Health and Welfare (2011)
South Africa	2010	Confirmed outbreak was reported to the OIE in February 2011. Information is available on WAHID website	EFSA Panel on Animal Health and Welfare (2011)
Canada	2010	Confirmed outbreak of EUS in a new susceptible species (brown bullhead, *Ameiurus nebulosus*) was reported to the OIE in March 2011. Information is available on WAHID website	EFSA Panel on Animal Health and Welfare (2011)

Appendix table II Bacterial pathogens of freshwater and marine fish (after Austin and Austin 2012).

Pathogen	Disease	Host range	Geographical distribution
Anaerobes			
'*Catenabacterium*' sp	–	Grey mullet (*Mugil auratus*), Red fish (*Sebastes* sp.)	USA
Clostridiaceae representative			
Clostridium botulinum	Botulism	Salmonids	Denmark, England, USA
Eubacteriaceae representative			
Eubacterium tarantellae	Eubacterial meningitis	Striped mullet (*Mugil cephalus*)	USA
Gram-Positive bacteria – the 'Lactic Acid' bacteria			
Carnobacteriaceae representative			
Carnobacterium maltaromaticum -like organism	–	Lake whitefish (*Coregonus clupeaformis*)	USA
Carnobacterium piscicola	Lactobacillosis, pseudokidney disease	Salmonids	North America, UK
Aerococcaceae representative			
Aerococcus viridans	–	Tilapia	China
Enterococcaceae representatives			
Enterococcus (*Streptococcus*) *faecalis* subsp. *liquefaciens*	–	Rainbow trout (*Oncorhynchus mykiss*), catfish	Italy
Vagococcus salmoninarum	Lactobacillosis, pseudokidney disease, peritonitis, septicaemia	Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), rainbow trout	Australia, France, North America, Turkey
Lactobacillaceae representative			
Lactobacillus spp.	Lactobacillosis, pseudokidney disease	Salmonids	North America, UK
Leuconostocaceae representative			
Weissella sp.	Haemorrhagic septicaemia	Rainbow trout	Brazil, China
Streptococcaceae representatives			
Lactococcus garvieae (=*Enterococcus seriolicida*)	Streptococcicosis/ streptococcosis	Many fish species	Australia, Brazil, Europe, Israel, Japan, Saudi Arabia, Red Sea, South Africa, Taiwan, USA
Lactococcus piscium	Lactobacillosis, pseudokidney disease	Rainbow trout	North America
Streptococcus dysgalactiae	Streptococcosis	Amur sturgeon (*Acipenser schrenckii*), amberjack (*Seriola dumerili*), Nile tilapia (*Oreochromis niloticus*), yellowtail (*Seriola quinqueradiata*)	Brazil, China, Japan

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
Streptococcus agalactiae (=*Str. difficilis*)	Meningo-encephalitis	Carp (*Cyprinus carpio*), grouper (*Epinephelus lanceolatus*), rainbow trout, silver pomfret (*Pampus argenteus*), tilapia (*Oreochromis* spp.)	Australia, Columbia, Israel, Kuwait, USA
Streptococcus ictaluri	Streptococcosis	Channel catfish	USA
Streptococcus iniae (*Str. shiloi*)	Acute septicaemia, meningoencephalitis, streptococcicosis/ streptococcosis	Various freshwater and marine fish species	Australia, Bahrain, China, Europe, Israel, Japan, Saudi Arabia, South Africa, USA
Streptococcus milleri	–	Koi carp (*Cyprinus carpio*)	UK
Streptococcus parauberis	Streptococcicosis/ streptococcosis	Turbot (*Scophthalmus maximus*)	Spain
Streptococcus phocae	Streptococcosis	Atlantic salmon	Chile
Aerobic Gram-Positive Rods and Cocci			
Renibacterium salmoninarum	Bacterial kidney disease (BKD; Dee disease; corynebacterial kidney disease)	Salmonids	Europe, Japan, North and South America
Bacillaceae representatives			
Bacillus spp.	Septicaemia; bacillary necrosis	Various freshwater fish species including catfish (*Pangasius hypophthalmus*)	Nigeria, Vietnam
Bacillus cereus	Branchio-necrosis	Carp (*Cyprinus* sp.), striped bass (*Morone saxatilis*)	USA
Bacillus mycoides	Ulceration	Channel catfish (*Ictalurus punctatus*)	Poland, USA
Bacillus subtilis	Branchio-necrosis	Carp	Poland
Corynebacteriaceae representatives			
Corynebacterium aquaticum	Exophthalmia	Striped bass	USA
Coryneform bacteria	'Corynebacteriosis'	Salmonids	England
Micrococcus luteus	Micrococcosis	Rainbow trout	England
Mycobacteriaceae representatives			
Mycobacterium spp. (*Myc. abscessus, Myc. anabanti, Myc. chelonei* subsp. *piscarium, Myc. fortuitum, Myc. gordonae, Myc. marinum, Myc. montefiorense, Myc. neoaurum, Myc. piscium, Myc. platypoecilus, Myc. poriferae. Myc. pseudoshottsii, Myc. ranae, Myc. salmoniphilum, Myc. shottsii, Myc. scrofulaceum, Myc. simiae, Myc. smegmatis, Myc. ulcerans*)	Mycobacteriosis (fish tuberculosis)	Most fish species	worldwide
Nocardiaceae representatives			
Nocardia spp. (*Noc. asteroides, Noc. salmonicida; Noc. seriolae*)	Nocardiosis	Most fish species	worldwide
Rhodococcus sp.	Ocular oedema	Chinook salmon (*O. tshawytscha*)	Canada

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
Rhodococcus erythropolis	?	Atlantic salmon	Norway, Scotland
Rhodococcus qingshengii	Peritonitis	Atlantic salmon	Chile
Planococcaceae representative			
Planococcus sp.	–	Salmonids	England
Staphylococcaceae representatives			
Staphylococcus aureus	Eye disease	Silver carp (*Hypophthalmichthys molitrix*)	India
Staphylococcus epidermidis	–	Gilthead sea bream (*Sparus aurata*), red sea bream (*Chrysophrus major*), yellowtail (*Seriola quinqueradiata*)	Japan, Turkey
Staphylococcus warneri	Ulcerations	Rainbow trout	Spain
Gram-Negative Bacteria			
Aeromonadaceae representatives			
Aeromonas allosaccharophila	–	Elvers	Spain
Aeromonas bestiarum	–		USA
Aeromonas caviae	Septicaemia	Atlantic salmon (*Salmo salar*)	Turkey
Aeromonas hydrophila (*=Aer. liquefaciens, Aer. punctata*)	Haemorrhagic septicaemia, motile aeromonas septicaemia, redsore disease, fin rot	Many freshwater fish species	worldwide
Aeromonas jandaei	–	Eel (*Anguilla* sp.)	Spain
Aeromonas salmonicida (subsp. *achromogenes, masoucida, salmonicida* and *smithia*) {*=Haemophilus piscium*}	Furunculosis, carp erythrodermatitis, ulcer disease	Salmonids, cyprinids, and marine species (dabs, cod)	worldwide
Aeromonas sobria	–	Garra rufa (*Garra rufa*), perch (*Perca fluvialitis*), gizzard shad (*Dorosoma cepedianum*), tilapia (*Oreochromis niloticus*)	China, Slovakia, Switzerland, USA
Aeromonas schubertii	Tuberculous lesions	Snakehead (*Ophiocephalus argus*)	China
Aeromonas veronii biovar sobria	Epizootic ulcerative syndrome, infectious dropsy	African catfish (*Clarias gariepinus*), rajputi (*Puntius gonionotus*), rui (*Labeo rohita*), catla (*Catla catla*), shole (*Channa striatus*), oscar (*Astronotus ocellatus*)	Bangladesh, India
Alteromonadaceae representatives			
Pseudoalteromonas piscicida	Egg disease	Damselfish	USA
Pseudoalteromonas undina	–	Sea bass, sea bream	Spain
Shewanella putrefaciens	Septicaemia	Rabbit fish (*Siganus rivulatus*)	Saudi Arabia
Campylobacteriaceae representative			
Arcobacter cryaerophilus	–	Rainbow trout	Turkey
Enterobacteriaceae representatives			
Citrobacter freundii	–	Salmonids, sunfish (*Mola mola*), carp (*Cyprinus carpio*)	Europe, India, USA

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
`Edwardsiella ictaluri Enteric septicaemia of catfish	Enteric septicaemia of catfish	Ayu, bagrid catfish (*Pelteobagrus nudiceps*), brown bullhead (*Amieurus nebulosus*), channel catfish, freshwater catfish (*Pangasius hypophthalmus*), danio (*Danio devario*), striped catfish (*Pangasius hypophthalmus*), yellow catfish (*Pelteobagrus fulvidraco*)	China, Indonesia, Japan, USA, Vietnam
Edwardsiella tarda (*Paracolobactrum anguillimortiferum, Edw. anguillimortifera*)	Redpest, edwardsiellosis, emphysematous putrefactive disease of catfish	Freshwater and some fish species	Japan, Spain, USA
Enterobacter cloacae	–	Mullet (*Mugil cephalus*)	India
Escherichia vulneris	Septicaemia	Various freshwater fish species	Turkey
Hafnia alvei	Haemorrhagic septicaemia	Cherry salmon (*O. masou*), rainbow trout	Bulgaria, England, Japan
Klebsiella pneumonia	Fin and tail disease	Rainbow trout	Scotland
Plesiomonas shigelloides	–	African catfish (*Heterobranchus bidorsalis*), eel, gourami (*Osphyronemus gourami*), rainbow trout, sturgeon (*Acipenser sturio*)	Germany, Portugal, Spain
Pantoea (*=Enterobacter*) *agglomerans*	–	Dolphin fish (*Coryphaena hippurus*)	USA
Providencia (*Proteus*) *rettgeri*	–	Silver carp	Israel
Salmonella enterica subsp. *arizonae* (*= Sal. Choleraesuis* subsp. *arizonae = Sal. arizonae*)	Septicaemia	Pirarucu (*Arapaima gigas*)	Japan
Serratia liquefaciens	Septicaemia	Arctic charr (*Salvelinus alpinus*), Atlantic salmon, turbot	France, Scotland, USA
Serratia marcescens	–	White perch (*Morone americanus*)	USA
Serratia plymuthica	–	Rainbow trout	Poland, Scotland, Spain
Yersinia intermedia	–	Atlantic salmon	Australia
Yersinia ruckeri	Enteric redmouth (ERM), salmonid blood spot	Salmonids	Australia, Europe, North and South America
Flavobacteriaceae representatives			
Chryseobacterium balustinum (*=Flavobacterium balustinum*)	Flavobacteriosis	Marine fish	USA
Chryseobacterium piscicola	Skin and muscle ulceration	Atlantic salmon, rainbow trout	Chile, Finland
Chryseobacterium scophthalmum (*=Flavobacterium scophthalmum*)	Gill disease; generalized septicaemia	Turbot	Scotland
Flavobacterium branchiophilum	Gill disease	Salmonids	Europe, Korea, Japan, USA
Flavobacterium columnare (*=Flexibacter/Cytophaga columnaris*)	Columnaris, saddleback disease	Many freshwater fish species	worldwide

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
Flavobacterium hydatis (=*Cytophaga aquatilis*)	Gill disease	Salmonids	Europe, USA
Flavobacterium johnsoniae (=*Cytophaga johnsonae*)	Gill disease, skin disease	Barramundi (*Lates calcarifer*), koi carp, rainbow trout, longfin eel (*Anguilla mossambica*)	Australia, France, South Africa
Flavobacterium oncorhynchi	–	Rainbow trout	Spain
	Bacterial gill disease	Salmonids	Europe, USA
Flavobacterium psychrophilum (=*Cytophaga psychrophila*)	Coldwater disease, rainbow trout fry syndrome, necrotic myositis	Perch (*Perca fluviatilis*), salmonids, sea lamprey (*Petromyzon marinus*)	Australia, Europe, Japan, North America
Tenacibaculum dicentrarchi	–	Sea bass	Spain
Tenacibaculum discolor	–	Sole (*Solea senegalensis*)	Spain
Tenacibaculum gallaicum	–	Turbot (*Psetta maxima*)	Spain
Tenacibaculum maritimum (=*Flexibacter maritimus*)	Bacterial stomatitis, gill disease, black patch necrosis	Many marine fish species	Europe, Japan, North America
Tenacibaculum ovolyticum (=*Flexibacter ovolyticus*)	Larval and egg mortalities	Halibut (*Hippoglossus hippoglossus*)	Norway
Tenacibaculum soleae	Tenacibaculosis	Sole (*Solea senegalensis*), wedge sole (*Dicologoglossa cuneata*), brill (*Scophthalmus rhombus*)	Spain
(‘*Cytophaga rosea*’)	Gill disease	Salmonids	Europe, USA
Sporocytophaga sp.	Saltwater columnaris	Salmonids	Scotland, USA
Francisellaceae representatives			
Francisella sp.	Granulomatous inflammatory disease	Atlantic cod (*Gadus morhua*), hybrid striped bass (*Morone chrysops* × *M. saxatilis*), three-line grunt (*Parapristipoma trilineatum*), tilapia	Costa Rica, Japan, Norway, USA
Francisella asiatica	Francisellosis	Tilapia, three-line grunt	Costa Rica, England, Japan
Francisella noatunensis (=*Fr. philomiragia* subsp. *noatunensis* = *Fr. piscicida*)	Francisellosis, visceral granulomatosis	Atlantic cod, Atlantic salmon,	Chile, Norway
Halomonadaceae representative			
Halomonas (=*Deleya*) *cupida*	–	Black sea bream (*Acanthopagrus schlegeli*)	Japan
Moraxellaceae representatives			
Acinetobacter sp.	Acinetobacter disease	Atlantic salmon, channel catfish	Norway, USA
Moraxella sp.	–	Striped bass	USA
Moritellaceae representatives			
Moritella marina (*V. marinus*)	Skin lesions	Atlantic salmon	Iceland
Moritella viscosa	Winter ulcer disease/ syndrome	Atlantic salmon	Iceland, Norway, Scotland
Mycoplasmataceae representative			
Mycoplasma mobile	Red disease	Tench (*Tinca tinca*)	USA

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
Myxococcaceae representative			
Myxococcus piscicola	Gill disease	Green carp (*Ctenopharyngodon idellus*)	China
Neisseriaceae representative			
Aquaspirillum sp.	Epizootic ulcerative syndrome	Snakeheads (*Ophicephalus striatus*) and catfish (*Clarias batrachus*)	Thailand
Oxalobacteraceae			
Janthinobacterium lividum	Anaemia	Rainbow trout	Scotland
Pasteurellaceae representative			
Pasteurella skyensis	?	Atlantic salmon	Scotland
Photobacteriaceae representatives			
Photobacterium damselae subsp. *damselae* (=*Photobacterium histaminum*)	Vibriosis	Damsel fish (*Chromis punctipinnis*), redbanded sea bream (*Pagrus auriga*) rainbow trout, sea bass (*Lates calcarifer*), sharks, turbot, yellowtail	Asia, Europe, USA
Photobacterium damselae subsp. *piscicida* (=*Pasteurella piscicida*)	Pasteurellosis, pseudotuberculosis	Bluefin tuna (*Thunnus thynnus*), gilthead sea bream (*Sparus aurata*), sole (*Solea senegalensis*), striped bass (*Morone saxatilis*), white perch (*Roccus americanus*), yellowtail	Europe, Japan, USA
Piscirickettsiaceae representative			
Piscirickettsia salmonis	Coho salmon syndrome, salmonid rickettsial septicaemia	Salmon, sea bass (*Atractoscion nobilis*)	Canada, Chile, Greece, Norway, Scotland, USA
Rickettsia-like organism	Red mark syndrome/ strawberry disease	Rainbow trout	UK, USA
Pseudomonadaceae representatives			
Pseudomonas anguilliseptica	Red spot (Sekiten-byo), winter disease	Rainbow trout, marine fish species, and particularly cod, eels (*Anguilla anguilla, A. japonica*), black spot sea bream (*Pagellus bogaraveo*), gilthead sea bream (*Sparus aurata*)	Finland, France, Japan, Portugal, Scotland, Spain
Pseudomonas baetica	–	Wedge sole (*Dicologoglossa cuneata*)	Spain
Pseudomonas chlororaphis	–	Amago trout (*Oncorhynchus rhodurus*)	Japan
Pseudomonas fluorescens	Generalised septicaemia	Most fish species	worldwide
Pseudomonas luteola	Generalised septicaemia	Rainbow trout	Turkey
Pseudomonas plecoglossicida	Bacterial haemorrhagic ascites	Ayu (*Plecoglossus altivelis*), pejerrey (*Odonthestes bonariensis*)	Japan
Pseudomonas pseudoalcaligenes	Skin ulceration	Rainbow trout	Scotland
Pseudomonas putida	Haemorrhagic ascites, ulceration	Ayu, rainbow trout	Japan, Turkey
Vibrionaceae representatives			
Aliivibrio fischeri	–	Gilthead sea bream , turbot	Spain
Ali. logei	Skin lesions	Atlantic salmon	Iceland

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
Ali. salmonicida	Coldwater vibriosis, Hitra	Atlantic salmon	Canada, Norway, Scotland
Vibrio aestuarianus	–	Tongue sole (*Cynoglossus semilaevis*)	China
V. alginolyticus	Eye disease, septicaemia	Cobria (*Rachycentron canadum*), gilthead sea bream, grouper (*Epinephelus malabanicus*), sea bream (*Sparus aurata*)	Asia, Europe, Israel
V. anguillarum (=Listonella anguillarum)	Vibriosis	Most marine fish species	worldwide
V. cholerae (non-01)	Septicaemia	Ayu, gold fish (*Carassius aurata*)	Australia, Japan
V. furnissii	–	Eel	Spain
V. harveyi (=*V. carchariae and V. trachuri*)	Eye disease (blindness), necrotising enteritis, vasculitis, granuloma	Gilthead sea bream, sea bass, common snook (*Centropomus undecimalis*), horse mackerel (*Trachurus japonicus*), milkfish, red drum *(Sciaenops ocellatus*), sharks (*Carcharhinus plumbeus, Negaprion breviorstris*), sole (*Solea senegalensis*), summer flounder (*Paralichthys dentatus*), tiger puffer (*Takifugu rubripes*)	Europe (notably Spain), Japan, Taiwan, USA
V. ichthyoenteri	Intestinal necrosis/enteritis	Japanese flounder (*Paralichthys olivaceus*), summer flounder, olive flounder	Japan, Korea, USA
V. ordalii	Vibriosis	Most marine fish species	worldwide
V. pelagius	–	Turbot	Spain
V. ponticus	Ulcerative disease	Japanese sea bass (*Lateolabrax japonicus*)	China
V. splendidus	Septicaemia, vibriosis	Corkwing wrasse (*Symphodus melops*), gilthead sea bream, turbot	Norway, Spain
V. tapetis	Vibriosis	Corkwing wrasse, ovate pompano (*Trachinotus ovatus*)	Norway
V. vulnificus	Septicaemia	Eel	Europe, Japan, P.R.C., USA
V. wodanis	Winter ulcer disease/ syndrome	Atlantic salmon	Iceland, Norway, Scotland
Miscellaneous pathogens			
'*Candidatus* Arthromitus'	Summer enteritic syndrome, Rainbow trout gastroenteritis	Rainbow trout	Croatia, France, Italy, Spain, UK
'*Candidatus* Branchiomonas cysticola'	Epitheliocystis	Atlantic salmon	Norway
'*Candidatus* Clavochlamydia salmonicola'	Epitheliocystis	Freshwater salmonids	North America, Norway
'*Candidatus* Piscichlamydia salmonis'	Epitheliocystis	Atlantic salmon	Norway
'*Candidatus* Renichlamydia lutjani'	"Epitheliocystis"-like	Blue-striped snapper (*Lutjanus kasmira*)	Hawaii, USA
Chlamydiales representative	Epitheliocystis	Leopard sharp (*Triakis semifasciata*)	Swiss aquarium
Streptobacillus	–	Atlantic salmon	Ireland

Appendix table II contd.

Pathogen	Disease	Host range	Geographical distribution
unidentified	Gill lesions	Rockfish	Japan
unidentified	*Varracalbmi*	Atlantic salmon	Norway
unidentified	Ulceration	Rainbow trout	Scotland

Note: Taxa (highlighted by quotation marks), namely '*Catenabacterium*', '*H. piscium*' and '*Myxobacterium*' are of doubtful taxonomic validity. Others, such as *Pr. rettgeri* and *Sta. epidermidis*, are of questionable significance in fish pathology insofar as their recovery from diseased animals has been sporadic.

Appendix III Apparatus used in this study (image source: internet).

Incubator (28~30°C)

Incubator (37°C)

Orbital shaker

Photoelectric colorimeter

Hot plate and Magnetic stirrer

Mini centrifuge

Hot water bath

UV transilluminator

Appendix III contd.

Table top centrifuge

Gel electrophoresis unit

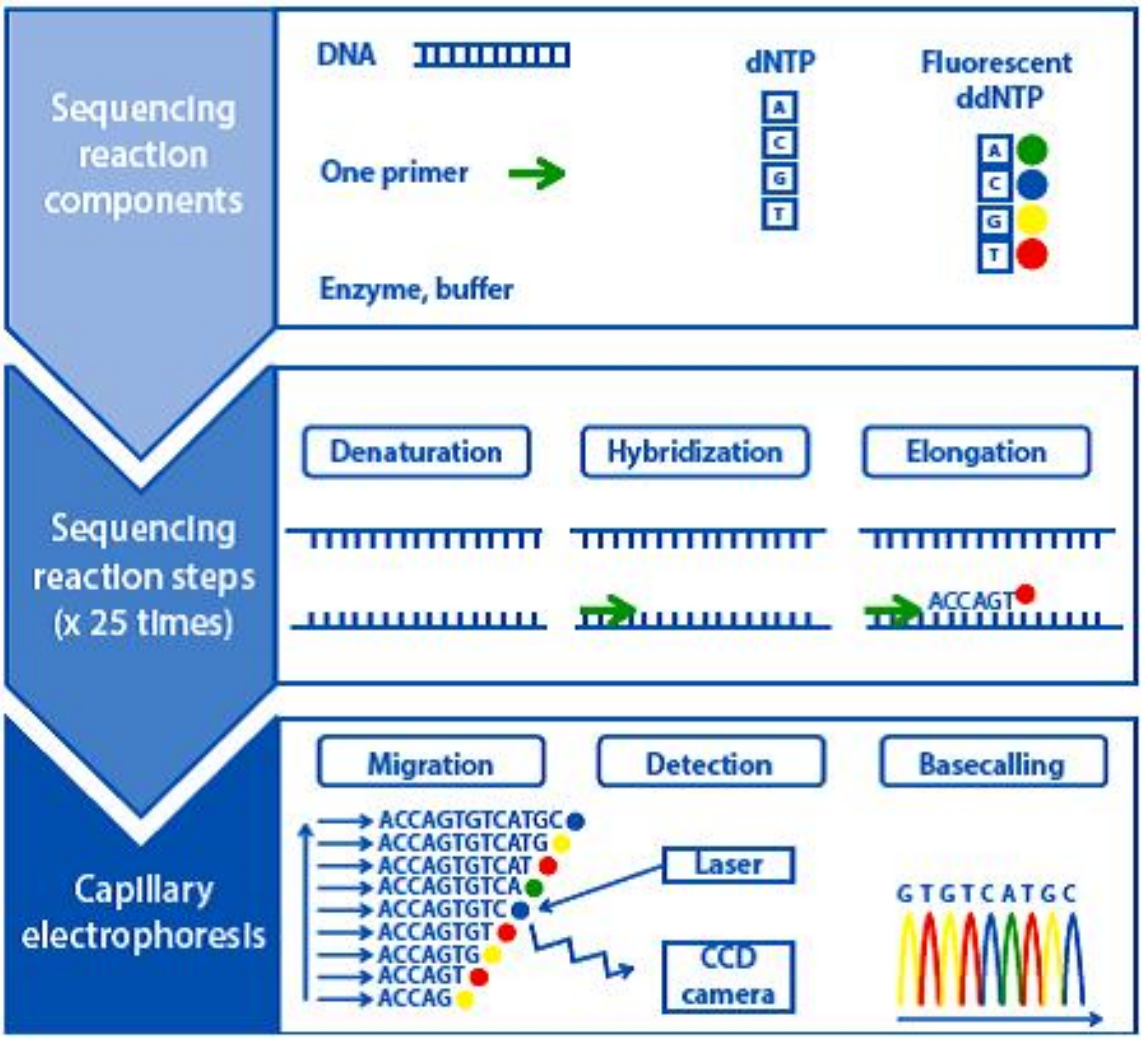
Thermal cycler

Gel documentation

Nanodrop spectrophotometer

Sequencer

Appendix IV Sanger sequencing workflow using dye terminator technology (source: internet).



Appendix V Obtained nucleotide sequences of the isolate CK (creamy colony forming bacteria isolated from mucilage of diseased carp fish from Kakanhat area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CCTTTAGGTCAGTCTACCGTCCGAGTCGGCGGTAGCACAGGAGTGCTTGCTCTCGGGTGA
CGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT
TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT
GCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCAGT
AAGGTTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC
GAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGGTGAAAT
GCGTAGAGATCTGGAGGAATACCCGGTGGCGAATGCGCCCCCTGACAAAGACTGACGCTC
AGTGCGAAAGCGTGGGGGAGCAAACAGATTAGAAACTCTGTGATGATATTACCAA

Appendix VI Obtained nucleotide sequences of the isolate WK (white colony forming bacteria isolated from diseased carp fish from Kakanhat area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGTAACGGGAGTCTACCATGCGGTCGACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA
GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA
ACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGC
CATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACG
ATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAG
GTTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG
CAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA
ACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT
AGAGATCTGGAGGAATACCGGTGGCGAAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGGAGCAAACAGGATAAGAAACTGTGTGGTTGATACAAAA

Appendix VII Obtained nucleotide sequences of the isolate YK (yellow colony forming bacteria isolated from diseased carp fish from Kakanhat area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGTTACGCAGTCTACCGTCCGCTGGAGGTAGCCAGAGTGGTTTGCTCCTCGGGTGAGGGT
GGCAGATGGGTGGTTAGTGTCGGGAGCTCTCCCAGATGGGGGGGATAACCTGTGAAAAGG
GGGGCTAATCCCGTATAACCTCACAACACCAAAGAGGGGCCCCTTCGGCCCTCTCTCTCT
CATAAGCCCCCATAGGGTTTTATCTAATGCGGGGGGTAGTGCCCCCCCTACGACAATATC
CCTATCTGGTCTGAGAAGAAGACCCCCCACTCTGGCTCTGACACACGGCCCACACTCCTA
CGGGGGGCCACTGTGGAAAATTTTGCACTGTGGGCGCACCCCTGATGCCCCCGTGCCGCG
TGTAAAAAAAGCGCCTTCGGGTTGAAATATTTTTTCCGCGGGGAAGAGGGGTGTGCGTAT
AATACCCGCCCCTAGTGATTTCCCCCGCAAAAAACACCCGGTCTATCTCCGTGCCCACCC
CCGCGGTTATAGAGAGGGTGCACGCTTATCTCAAATTTACTGGGCATAGAGCACACGCGC
GCTCTCTGTTAACTCATATGTAATCTCCCCGGTTTTACCCTGGAATCTGCTTTTGACTCT
GGGGGCGCTTGACTCCTTATAGGGGGGGGATAAATTTCCGGGTGTCGCTGAAAAGTGCAT
AGATCTCTAGAAAAAAACCCCTGTCGCAGGGGGGGGCCCCCTCGTGGAAAAAACTACACC
ACTCCTCATGTGAAAAAAGCGGTGGGAGCGCACAACGGAGTATTAGAACCCTTTTTTTTC
TCAACAAA

Appendix VIII Obtained nucleotide sequences of the isolate CD (creamy colony forming bacteria isolated from diseased carp fish from Darusha area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CATTACCGGCAGTCTACAGTGCGAGTCGGCGGTAGCGGGGAGATGTAGCTTGCTACTTTT
GCCGGCGAGCGCGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGGCGAGGGGGATAA
CTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGG
GCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACC
AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGC
AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAA
AGGTCGGTAGCTAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATT
GCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCCATGTGTAGCGG
TGAAATGCGTAGAGATCTGGGAGGATACCGGTGGCGAAGGCGCCCCTGGACAAAGACTGA
CGCTCAGTGCGAAGCGTGGGGGAGCAAACATGATTAGAACGCTGCTGTGTGTATATCACA
GG

Appendix IX Obtained nucleotide sequences of the isolate YD (yellow colony forming bacteria isolated from diseased carp fish from Darusha area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

GCTTGCGGCGCTATAATGCAGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGA
GTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATAACATGGGGAAACTCATGCTAATAC
CGGATAGGGTCTTCTCTCGCATGAGAGGAGACGGAAAGGTGGCGCAAGCTACCACTTACG
GATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG
AACGATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGACGAACAAGTACCGTTCGA
ACAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAACCCCGGTTCGCATCGGAAACTGT
GTAGCTTGAGTGCAGAAGAGGAAAGCGGTATTCCACGTGTAGCGGTGAAATGCGTAGAGA
TGTGGAGGACACCAGTGGCGAAGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAA
GCGGTGGGGAGCAAACAGATTAGAACGTCTGCTGATGATGAGATCCAA

Appendix X Obtained nucleotide sequences of the isolate CYD (creamy-yellow colony forming bacteria isolated from diseased carp fish from Darusha area, Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

GGTGGGGCATCTATAATGCAGTCGAGCGAGTCGCTTCGGGGGCTAGCGGCGGACGGGTGA
GTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATAACATGGGGAAACTCTTGCTAATAC
CGGATAGGGTCTTCTCTCGCATGAAATGAGACGGAAAGGTGGCGCAAGCTACCACTTGCT
GATGGGCCTGCTGCGCATTAACTAGTTGGGGGGGTAACGGCCTACCAAGGCGACGATGCC
TAACCCACCTGAAAGGGTGACCGGCCACACTGGGACTGACACACCGGCCACACTCCTACG
GGAGGCACTCCTACGGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG
AACGATGAACGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGACTAACAAGTACCGTTCGA
ACAGGGCGGTACCTTGACGGTACCTGACTAGAAAGCCACGGCTAACTACGTGCCAGCAAC
CGCGGTAATACGTACGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CGGCTATGTAAATCTGGTGTTATAGCCCTGGGCTCAACCCCCGGCTGCGTATCCCAGACT
GTGTAACTTGAGTGCAGAAGACGTAAAGCTGCTATATCCACGTGTAGCGATTTACATGCG
TAGAGATTGTGGAGGCAACACCATTGGCGAAAGCAGCTTTCTCGTCTGTACTGACTCTGT
ATGTACTAAGCGCTGGAGGAGCAAACGATGAGATACGCCGGGGATATTCACACCCGG

Appendix XI Obtained nucleotide sequences of the isolate SW/Cr (creamy colony forming bacteria isolated from pond water from Ullapara, Sirajganj) from ABI Prism 3130 genetic analyzer in FASTA format.

CCTAACCGGGGCCCTAACCGTCCAAGTCGAACGGTAGCACAGGAGAGCTTGCTCTCGGGT
GACGAGTGGCGGAGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT
GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC
TTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA
TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGT
TGAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
CACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT
CGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT
GCGTAGAGATCTGGGAGGAATACCCGGTGGCGAAGGCGGCCCCTGGACAAAGACTGACGC
TCATGGTGCGAAAGCGTGGGGGGAGCAAACAGGATTAGATACTCTTGTTGAGTATCTCAA
AT

Appendix XII Obtained nucleotide sequences of the isolate SW/Yel (yellow colony forming bacteria isolated from pond water from Ullapara, Sirajganj) from ABI Prism 3130 genetic analyzer in FASTA format.

CTATGGGGGCCGTCTATGATGCAGTCGAGCGGATCGATGGGAGCTTGCTCCCTGAGATCA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA
AACCGGGGCTAATACCGGATAACACCTACCCCCGCATGGGGGAAGGTTGAAAGGTGGCTT
CGGCTATCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG
GAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGA
ACAAGTGCCGTTCGAATAGGGCGGCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCG
GTGAAATGCGTAGATATTTGGAGGAACACCAGTGGCGAAGCGACTTTCTGGTCTGTAACT
GACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGAACGCGTGCGTATGTATTC
ACCAA

Appendix XIII Obtained nucleotide sequences of the isolate GSH/Cr (creamy colony forming bacteria isolated from gill, skin and head surface of diseased *Catla* from Ullapara, Sirajganj) from ABI Prism 3130 genetic analyzer in FASTA format.

TATAGGCGAGAGTCCTCACAGATGCGAGTCGGCGGCAGCGGGGAGAGTAGCTTGCTACTT
TTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATA
ACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCG
GGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATG
CAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGA
AAGGTTGGTAGCTAATAACTGCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAAT
TGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGCCCCCTGGACAAAGACTG
ACGCTCAAGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGAAACGCGTGTGTGATAACC
AAAAG