ENVIRONMENTAL FACTORS AFFECTING THE PATHOGENESIS OF *Edwardsiella ictaluri* IN STRIPED CATFISH *Pangasianodon hypophthalmus* (Sauvage)

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Declaration

I hereby declare that the work and results presented in this thesis has been composed in its entirety by me. Except where specifically acknowledged, the work described in this thesis has been conducted by me and has not been submitted for any other degree or qualification.

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ABSTRACT

Bacillary Necrosis of Pangasius (BNP) caused by *Edwardsiella ictaluri* is considered to be the most serious disease occurring in farmed striped catfish (*Pangasianodon hypophthalmus*) in Vietnam. This disease has had an increasing impact over the last ten years and has been reported to cause 50-90% mortality of stocks during a single outbreak. Data obtained from natural outbreaks of *E. ictaluri* in striped catfish showed the role of environmental factors in the establishment and progression of this disease. At present, factors affecting the virulence and transmission of *E. ictaluri* in striped catfish are poorly understood. The central hypothesis of this thesis focuses on the complex picture of the environmental factors and infectivity of *E. ictaluri* in striped catfish.

In this study, 80 isolates of *E. ictaluri* recovered from natural clinical disease outbreaks occurring in striped catfish farms between 2002 and 2011 located in 4 distinct geographical areas within Vietnam were characterised using a variety of methods. The biochemical profiles showed that *E. ictaluri* isolates from striped catfish in Vietnam have similar phenotypic characteristics to other *E. ictaluri* isolates from other infected fish species. These data showed high levels of phenotypic homogeneity between the *E. ictaluri* isolates investigated. The status of isolates recovered from natural infections over time and from geographically distinct farms was evaluated using pulsed-field gel electrophoresis (PFGE), plasmid profile identification and antibiotic sensitivity tests. The PFGE results showed 6 main groups with a similarity of 82% and the corresponding genotypes of the prevalent isolates illustrated annual differences. Three plasmid

groups were identified distributed among the isolates investigated, in which high molecular weight plasmids of approximately 35 and 140 kb were found in two of the groups. Plasmid profiles of the present study did not show any trend of geographical region or year of isolation. The 140 kb plasmid has been considered as a multi-antibiotic resistance plasmid which confers resistance to tetracycline, trimethoprim and sulphonamides. All Vietnamese isolates showed a high level of resistance to Oxolinic acid, Sulfadimethoxine/Ormetoprim (Romet), Oxytetracycline and Amoxicillin.

A reproducible bacterial immersion challenge model was developed and the LD_{60} estimated prior to performing subsequent experimental challenge studies. Fish were exposed to 10^7 cfu ml^{-1} of *E. ictaluri* by immersion for up to 30 seconds, resulting in a cumulative percentage mortality of 63%. *Edwardsiella ictaluri* was recovered and identified from all the dead and moribund fish during these experiments and affected fish showed similar clinical signs and pathology to those reported from natural *E. ictaluri* infections. The present study resulted in a successful experimental immersion challenge model for *E. ictaluri* infection in healthy striped catfish. Cohabitation challenges were also developed and produced 15-40% mortality, typical clinical signs and pathology, and successful recovery of the challenge organism demonstrating horizontal transmission of *E. ictaluri* in striped catfish.

Experimental studies were then conducted to investigate the association between pH or salinity of water and susceptibility to *E. ictaluri* infection in striped catfish. The first experiments were performed in *in vitro* conditions in which *E. ictaluri* isolates were

cultured in a variety of pH and salt concentrations.   *In vivo* experiments were then designed where striped catfish were exposed to 10^7 cfu ml^{-1} of *E. ictaluri* for 30 seconds and then held at 4 different water pHs (5.5, 6.5, 7.5 and 8.5) or NaCl concentrations (0, 0.5, 1 and 1.5%). The results of *in vitro* experiments showed that a pH value between 5.5 to 6.5 and salt concentration between 0-0.5% were optimal for the growth of *E. ictaluri*. The *in vivo* experiments demonstrated that the cumulative mortality of striped catfish in water at pH 5 and pH 6 was significantly higher than that of fish maintained in more alkaline water ($p<0.05$). By contrast, the cumulative mortality of the striped catfish maintained in 0.5% salt concentration was significantly lower than those kept in 0%, 1% and 1.5% salt concentration ($p<0.05$). Clinical signs, lesions and histopathological changes in the affected fish were consistent with those reported in natural infections. This study highlighted the use of pH 8.5 and salinity of 0.5% NaCl as a means of decreasing the susceptibility of striped catfish to *E. ictaluri*.

In conclusion, this study used a variety of methods in order to enhance the understanding of the biochemical, biophysical characteristics, plasmid profile and antibiotic resistance as well as the relatedness of *E. ictaluri* isolates recovered from farmed striped catfish in Vietnam. This study provided two reliable and reproducible bacterial challenge models (immersion and cohabitation) and emphasised the link between pH and salinity with the infectivity and pathogenicity of *E. ictaluri* in striped catfish.

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µm	Micrometre
AFLP	Amplified fragment length polymorphism
AML	Amoxicillin
ARF	Aquaculture Research Facility
ATCC	American Type Culture Collection
BA	Blood Agar
BHI	Brain heart infusion
BNP	Bacillary Necrosis in Pangasius
bp	Base pairs
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DNTP	Deoxynucleotide triphosphate
DPC	Day post challenge
EDTA	Ethylenediaminetetraacetic acid
EIA	*E. ictaluri* agar
ELISA	Enzyme-linked immunosorbent assay
EMB	Eosin-Methylene Blue
Esa	*Edwardsiella* secretion apparatus (T3SS-related)
ESC	Enteric Septocaemia of catfish
Esc	*Edwardsiella* secretion chaperone (T3SS-related)
Ese	*Edwardsiella* secreted effector (T3SS-related)
Esr	*Edwardsiella* secretion regulator (T3SS-related)
et al.	Et alia (and others)
FAMEs	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organisation
h	Hours
i.p.	intraperitoneal
IFA	Indirect fluorescent antibody
IMM	Immersion
IOA	Institute of Aquaculture, University of Stirling
ISA	Iso-sensitest agar
ISB	Iso-sensitest broth
kb	Kilobase
L	Litres
LAMP	Loop-mediated isothermal amplification
LB	Luria Broth
LC_{60}	lethal concentration affecting 60% of the exposed population
LD_{60}	lethal dose affecting 60% of the exposed population
LDC	Lysine decarboxylase
MARD	Ministry of Agriculture and Rural Development of Vietnam
MIC	Minimum Inhibitory concentration

mg	Milligram
min	Minute
ml	Millilitre
MLST	Multilocus sequence typing
mM	Millimole
NBF	Neutral buffered formalin
NCIMB	National Collection of Industrial and Marine Bacteria
No.	Number
O LPS	O Lipopolysaccharide
O/F	Oxidation/Fermentation
OA	Oxolinic acid
OD	Optical density
ODC	Ornithine decarboxylase
OT	Oxytetracycline
PCR	Polymerase chain reaction
pEI1	*Edwardsiella ictaluri* plasmid 1
pEI2	*E. ictaluri* plasmid 2
per se	by itself
PFGE	Pulsed-field gel electrophoresis
pmol	Picomole
Rep-PCR	Repetitive extragenic palindromic - PCR
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
sec	Seconds
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
TE	Tris- Ethylenediaminetetraacetic acid
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UK	United Kingdom
USA	United States of America
V	Volt
V/v	Volume/volume

Chapter 1. GENERAL INTRODUCTION AND LITERATURE REVIEW.

Edwardsiella ictaluri, A MAJOR SIGNIFICANT PATHOGEN IN STRIPED

CATFISH *Pangasianodon hypophthalmus* (Sauvage 1878)

1.1. *Edwardsiella ictaluri* as a bacterial pathogen.

The genus *Edwardsiella* was first described in 1965 to comprise a group of enteric bacteria, with *Edwardsiella tarda* as the type species (Ewing et al. 1965). A second species, *E. hoshinae* was isolated from reptiles and birds and characterized by (Grimont et al. 1980). A distinct new species named *E. ictaluri* (Hawke et al. 1981) was isolated and characterized from diseased cultured farmed channel catfish and this study reported that *Edwardsiella ictaluri* was more closely related to *E. tarda* than to other members in the family *Enterobacteriaceae* based on DNA-DNA hybridization. *Edwardsiella ictaluri* is considered the most serious bacterial pathogen for the channel catfish industry in the USA (Plumb 1999; Plumb & Hanson 2011) and striped catfish farming in Vietnam (Phuong & Oanh 2010; De Silva & Phuong 2011; MARD 2011; Luu 2013; MARD 2013). The relationship between the American and Vietnamese strains of *E. ictaluri* is unclear although attempts have been made to investigate this (Bartie et al. 2012). The bacterium *E. ictaluri* is considered a homogeneous organism (Reid & Boyle 1989; Plumb & Vinitnantharat 1989; Lobb et al. 1993; Nagai et al. 2008; Sakai et al. 2009a; Bartie et al. 2012; Rogge et al. 2013), both in biochemical studies and through genomic fingerprinting using amplified fragment length polymorphism analysis (Sakai et al. 2009a). However, rep-PCR genomic fingerprinting or macro-restriction analysis results (Bartie et al. 2012; Rogge et al. 2013) have suggested geographical divergence

of the Asian and American isolates, and as such consider that *E. ictaluri* originating in Vietnam constitutes a unique genetic group compared with the USA isolates. A recent comparison of the Vietnamese and USA isolates of *E. ictaluri* recovered from clinical outbreaks of Vietnamese striped catfish and USA channel catfish (Rogge et al. 2013) showed that Vietnamese *E. ictaluri* isolates did not cause disease in the USA channel catfish. This demonstrates some interesting differences between the USA and Vietnamese isolates in terms of infectivity and host-pathogen specificity.

1.1.1. Bacterial identification and characterization.

Edwardsiella ictaluri is described as an intracellular organism belonging to the family *Enterobacteriaceae* and is a short, Gram-negative rod measuring about 0.75 x 2.5 μm when grown at 26°C (Hawke 1979) or 5 to 7 μm at 37°C (Plumb & Vinitnantharat 1989). This organism is weakly motile and able to produce peritrichous flagella at 25°C or 30°C (Hawke et al. 1981; Waltman et al. 1986; Plumb & Vinitnantharat 1989; Thune et al. 1993; Yuasa et al. 2003; Seçer et al. 2004; Zhang & Arias 2007; Ye et al. 2009) whereas the Vietnamese strains are described as weakly or non-motile at 28°C (Crumlish et al. 2002; Crumlish & Dung 2006; Crumlish et al. 2010; Hawke et al. 2013). It is cytochrome oxidase negative and weakly reactive biochemically, with positive reactions for lysine decarboxylase, ornithine decarboxylase, and oxidation and fermentation of glucose (Hawke et al. 1981; Plumb & Vinitnantharat 1989).

Edwardsiella ictaluri does not produce H$_2$S from triple sugar iron agar, which readily distinguishes it from *E. tarda*. This bacterium is also found to be negative for urease, indole, citrate, protease, esterase, pectinase, chitinase, lipase, alginase, collagenase

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and hyaluronidase (Hawke 1979; Waltman et al. 1986). The bacterium is not able to grow with NaCl concentrations higher than 1.5% and optimum growth temperature for *E. ictaluri* is considered to be between 25°C and 30°C (Waltman et al. 1986; Plumb & Vinitnantharat 1989). *E. ictaluri* is a β-haemolytic organism with 97% of the USA isolates studied being positive for haemolysin activity (Waltman et al. 1986). However, haemolysin activity in *E. ictaluri* is weaker than that in other species of *Enterobacteriaceae* (Janda et al. 1991) and haemolysin genes are homologous to the *Serratia* family (Williams & Lawrence 2005). *E. ictaluri* iron-regulated haemolysin is encoded by *eihA* and *eihB*, and is cell associated (Williams & Lawrence 2005). This haemolysin activity produces limited zones of haemolysis when cultured on blood agar plates because of the low diffusion of the high molecular weight haemolysin (Braun et al. 1987; Williams & Lawrence 2005).

All *E. ictaluri* isolates recovered from clinical outbreaks of disease in USA channel catfish contain plasmids (Speyerer & Boyle 1987; Newton et al. 1988; Reid & Boyle 1989; Fernandez et al. 2001; Rogge et al. 2013). The plasmid profile of *E. ictaluri* isolated from channel catfish in the USA consists of pEI1 (4.8 kb) and pEI2 (5.6 kb) (Fernandez et al. 2001) and is homogeneous for all isolates (Lobb & Rhoades 1987; Speyerer & Boyle 1987; Newton et al. 1988; Lobb et al. 1993). Non-channel catfish *E. ictaluri* isolates contain the 5.6 kb plasmid and the 4.0 kb plasmid derived from a mix of both pEI1 and pEI2 (Reid & Boyle 1989). Dung et al. (2009) also found plasmids in the Vietnamese *E. ictaluri* strains studied and described a high molecular weight plasmid (140 kb) belonging to the inc*K* group considered to mediate tetracycline resistance among *E. ictaluri* isolates from diseased striped catfish in Vietnam. Recently,

Bartie et al. (2012) defined another high molecular weight plasmid of 35 kb that distinguishes them from the plasmid profile of the USA isolates. This study also confirmed a 140 kb plasmid-mediated determinant responsible for combined tetracycline and sulphonamide resistance among *E*. *ictaluri* isolates within the Mekong Delta which was in agreement with previous studies from the same farming location (Dung et al. 2008; Dung et al. 2009). The high molecular weight plasmid of approximately 35 kb needs to be further studied to confirm this apparent association. By contrast, among 19 Vietnamese *E. ictaluri* isolates examined, Rogge et al. (2013) did not find any isolates containing these two high molecular weight plasmids and only described 4 plasmid profiles consisting of small molecular weight plasmids (approximately 4.0, 5.6 and 9.0 kb) in Vietnamese isolates that each differed from the profile of the USA isolates. None of the Vietnamese isolates carried a 4.8 kb plasmid. Amongst the 19 isolates investigated by Rogge et al (2013), 13 had only 1 small plasmid of approximately 4 kb, 1 isolate had only 1 plasmid of approximately 5.6 kb, 4 isolates had 2 plasmids of approximately 4 kb and 5.6 kb, and the final isolate had 2 plasmids of 4 kb and approximately 9 kb (Rogge et al. 2013). However, sequencing these plasmids showed that all Vietnamese isolates contained derivatives of the *E. ictaluri* plasmid pEI1. Rogge et al. (2013) also demonstrated from their study that the antibiotic resistance patterns in their isolates did not correlate to the presence or absence of any particular plasmids, including the >10 kb plasmid.

Various approaches were carried out to identify and distinct between strains of this bacterium by biochemical, biophysical characterization test, serological homogeneity and 16s RNA sequencing methods (Waltman et al. 1986; Newton et al. 1988; Plumb &

Vinitnantharat 1989; Bertolini et al. 1990; Lobb et al. 1993; Klesius et al. 2003; Panangala et al. 2005; Deng et al. 2008; Hassan et al. 2010; Liu et al. 2010). Molecular typing is important tool to study the genetic diversity. However, this typing method needs to be validated following convenienc criteria and performance criteria such as: typeability, reproducibility, stability, discriminatory ability, and concordance (Struelens 1996; appendix 1). The arbitrarily primed polymerase chain reaction (Ap-PCR) was used for fingerprinting and subtype *E. ictaluri* isolated from diseases channel catfish (Bader et al. 1998). Nagai et al. (2008) was completely described the phenotype and genotype characteristic of *E.ictaluri* in Japanese disease wild ayu by the partial nucleotide sequencing analysis of genes of 16S rRNA, type 1 fimbrial gene (*etfA*) and a heat shock protein gene (*dnaJ*). Recently, amplified-fragment length polymorphism (AFLP) analysis, a highly sensitive and reproducible technique for bacterial genotyping was used to clarify relationship among *Edwarsiella ictaluri* isolates from different geographic locations in Japan (Sakai et al. 2009a). Pulsed Field Gel Electrophoresis (PFGE) has been applied for typing of *E. ictaluri* isolates originating from the USA and Vietnam. These studies gave the evidence for homogeneity existing among isolates of *E.ictaluri* from different geographic location (see chapter 2).

1.1.2. Geographic distribution and host species of *E. ictaluri*.

While *E. tarda* is considered to have a broader host range (Thune et al. 1993), *E. ictaluri* in contrast has more narrow species susceptibility (Hawke et al. 2013). The geographic and host species of *E. ictaluri* are listed in Table 1.1.

Table 1. 1. Host species, habitat including aquarium (A), cultured (C), wild (W), experimental (E) and countries in which *Edwardsiella ictaluri* has been reported in the scientific literature.

Fish species	Habitat	Country	References
Ictaluridae			
Channel Catfish *Ictalurus punctatus*	C/W	USA	(Hawke 1979; Hawke et al. 1981)
White Catfish *Ameiurus catus*	W	USA	(Hawke et al. 1981)
Brown Bullhead *Ameiurus nebulosus*	W	USA	(Hawke et al. 1981)
Blue catfish *Ictalurus furcatus*	E	USA	(Wolters & Johnson 1994)
Tadpole madtom *Noturus gyrinus*	W	USA	(Klesius et al. 2003)
Claridae			
Walking catfish *Clarius batrachus*	W	Thailand	(Kasornchandra et al. 1987)
Pangasiidae			
Striped catfish	C	Vietnam	(Crumlish et al. 2002)
Pangasianodon hypophthalmus	C	Indonesia	(Yuasa et al. 2003)
Siluridae			
European catfish *Silurus glanis*	E	USA	(Plumb & Hilge 1987)
Southern catfish *Silurus meridionalis (Chen)*	C	China	(Geng et al. 2013)
Other families			
Green knifefish *Eigenmannia virescens*	A	USA	(Kent & Lyons 1982)
Blue tilapia *Oreochromis aureus*	E	USA	(Plumb & Sanchez 1983; Geng et al. 2013)
Bengal danio *Danio devario*	A	USA	(Waltman et al. 1985)
Chinook salmon *Oncorhynchus tshawytscha*	E	USA	(Baxa-Antonio et al. 1992)
Rainbow trout *Oncorhynchus mykiss*	E	USA	(Baxa-Antonio et al. 1992)
Striped bass *Morone saxatilis*	E	USA	(Baxa et al. 1990)
White perch *Morone americana*	E	USA	(Pasnik et al. 2007)
Zebra fish *Danio rerio*	E/A	USA	(Petrie-Hanson et al. 2007; Hawke et al. 2013)
Japanese eel *Anguilla japonica*	C	Taiwan	(Chung H.Y & Kou G.H 1983)
Rosy barb *Puntius conchonius*	A	Singapore	(Humphrey et al. 1986)
Ayu *Plecoglossus altivelis*	W	Japan	(Sakai et al. 2008; Nagai et al. 2008)
Yellow catfish *Pelteobagrus fulvidraco*	W/C	China	(Ye et al. 2009; Liu et al. 2010)
European sea bass *Dicentrarchus labrax*	C	Spain	(Blanch et al. 1990)
Rudd *Scardinius erythrophthalmus hesperidicus* H.	W	Croatia	(Topić Popović et al. 2001)
Tilapia *Oreochromis niloticus*	C	West Indies	(Soto et al. 2012)

Data of this table is modified and updated from Woo & Bruno (2011)

Since the first report of *E. ictaluri* infection in channel catfish (Hawke et al. 1981), this bacterium has been isolated from numerous freshwater fishes in a variety of habitats throughout the world related to the development of aquaculture as well as the spread of the pathogen.

1.1.3. Pathology

Edwardsiella ictaluri was first identified as the aetiological agent of Enteric Septicaemia of Catfish (ESC) by Hawke (1979) and it has been reported as the most serious pathogen affecting catfish farming in the USA (Hawke et al. 1998). In channel catfish, *E. ictaluri* infection can be manifested in two forms: acute and chronic presentations of the disease. In the acute form, affected fish show an acute gastrointestinal septicaemia associated with rapid mortality, whereas the chronic form was characterized by the external presence of a ''hole in the head'' lesion (Shotts et al. 1986), but both the acute and chronic stages are described as ESC. Diseased fish often hang with a head-up-tail-down posture and exhibit spinning or irregular swimming behaviour (Hawke 1979; Hawke et al. 1981). The brain is swollen (Newton et al. 1989; Morrison & Plumb 1994) but the presence of external lesions and petechial haemorrhages on skin, pale gills, as well as exophthalmia, which are often considered as characteristic for the disease (Hawke 1979). Internally, the peritoneal cavity may contain bloody ascitic fluid, and the intestine may exhibit petechial haemorrhages (Hawke 1979). Multifocal necrosis of the liver and swollen trunk kidney can be observed. Microscopically, hepatocytes are swollen and vacuolated and the exocrine

pancreas around hepatic vessels is necrotic (Shotts et al. 1986; Newton et al. 1989; Baldwin & Newton 1993; Morrison & Plumb 1994).

Ferguson *et al*. (2001) first described the pathology observed in clinically diseased striped catfish (*Pangasianodon hypophthalmus*) as severe multi-focal necrotizing and granulomatous bacterial infection and named the disease as Bacillary Necrosis of Pangasius (BNP). Crumlish et al. (2002) identified *E. ictaluri* as the aetiological agent of BNP where the affected fish displayed none of the external or internal clinical signs associated with ESC in the channel catfish. Instead fish suffering from BNP exhibit reducing appetite, spinning swimming behaviour, occasional petechial haemorrhages in the fins and eyes and fish may have pale colour of the skin and gills (Crumlish & Dung 2006; Crumlish et al. 2010). Internally, the presence of numerous, white, nodules or lesions, which are histologically described as multi-focal areas of cellular necrosis and granulomatous response, are visible throughout the viscera, but very evident to the naked eye in the spleen, liver and kidney of affected striped catfish, are considered as typical clinical signs of BNP (Ferguson et al. 2001; Crumlish et al. 2002; Dung et al. 2004; Crumlish & Dung 2006; Crumlish et al. 2010).

1.1.4. Transmission

It is known from USA studies on *E. ictaluri* that this bacterium is able to survive in the water column and the mud for many days and can establish and cause disease in a suitable host in the right conditions (Plumb & Quinlan 1986). Farm nets and equipment contaminated with *E. ictaluri* as well as infected fish carcasses left in the

pond were also considered as a means of *E. ictaluri* transmission within farming systems (Plumb & Quinlan 1986). It is believed that *E. ictaluri* is transmitted horizontally by intestinal infection arising from *E. ictaluri* infection in convalescent fish by their shedding of this bacterium into the water (Shotts et al. 1986). The gut and nares are considered sites of entry and primary infection in natural outbreaks of *E. ictaluri* in channel catfish (Shotts et al. 1986; Newton et al. 1989). Furthermore, *E. ictaluri* can be transmitted to susceptible channel catfish from fish that died from *E. ictaluri* infection through cannibalism of dead fish infected with this bacterium (Klesius 1994). The introduction of *E. ictaluri* infected fish into a pond containing healthy fish, or stocking healthy fingerlings into a pond containing older catfish that are carrying *E. ictaluri*, can result in the perpetuation and spread of *E. ictaluri* infection (Hawke et al. 1998). Although Hawke et al. (1998) suggested that fish that survive an outbreak of *E. ictaluri* can carry the bacterium in the kidney and liver for extended periods (up to 200 days), there is no evidence of the presence of *E. ictaluri* in striped catfish which have recovered from BNP outbreaks (Crumlish per. comm.)

1.1.5. Virulence and pathogenicity

The pathogenicity of *E. ictaluri* is dependent on its ability to infect and replicate within host cells (Booth et al. 2006) and the entry, survival and replication of *E. ictaluri* within neutrophils (Miyazaki & Plumb 1985; Ainsworth & Dexiang 1990), macrophages and cell lines (Shotts et al. 1986; Baldwin & Newton 1993; Booth et al. 2006; Dung et al. 2012) have been investigated in channel catfish. However, the virulence factors and mechanisms of pathogenesis of *E. ictaluri* have not been well characterized.

Chondroitinase activity, which is implicated in cartilage erosion of the frontal bone in the chronic "hole-in-the-head" lesion in channel catfish, has been considered as one of the virulence factors of *E. ictaluri* (Waltman et al. 1986; Stanley et al. 1994). The chondroitinase activity in virulent isolates of *E. ictaluri* was higher than in avirulent isolates (Stanley et al. 1994). Cooper II et al. (1996) found that *E. ictaluri* mutants which were deficient in chondroitinase activity produced by transposon mutagenesis showed the ability to induce an immune response that provided protection in channel catfish against challenge with *E. ictaluri*.

O Lipopolysaccharide (O LPS), which is apparently involved in attachment, has also been associated with the pathogenesis of *E. ictaluri*. A rifampicin-resistant strain of *E. ictaluri* which was reported to be associated with loss of O side chains was attenuated and proved effective when used as a live attenuated vaccine (Klesius & Shoemaker 1999). Lawrence et al. (2001) found that loss of O LPS side chains in *E. ictaluri* was associated with loss of virulence. The O LPS mutant had a significant decrease in its ability to survive in normal catfish serum but it retained the ability to resist killing by catfish neutrophils compared with wild-type *E. ictaluri* (Lawrence et al. 2003).

Using signature-tagged mutagenesis in a waterborne infection model, Thune et al. (2007) identified 50 transconjugants carrying transposon insertions in genes required for infection and survival of *E. ictaluri* in catfish. Three of those mutants had insertion genes involved in lipopolysaccharide biosynthesis, three genes involved in type III secretion systems (T3SS), and two in genes involved in urease activity. The T3SS

apparatus, encoded by *Edwardsiella* secretion apparatus (*esa*), chaperone (*esc*), effector (*ese*), and regulatory (*esr*) genes (Table 1.2), is able to translocate effector molecules directly from the bacterial cytosol to the channel catfish head kidney-derived macrophage cytoplasm. Mutation of the apparatus genes resulted in an inability to replicate in catfish cells and loss of virulence indicating that the T3SS is essential to the pathogenesis of *E. ictaluri* (Thune et al. 2007; Rogge & Thune 2011). In addition, the *E. ictaluri* plasmids, pEI1 and pEI2, encode proteins with similarity to T3SS effector proteins of other pathogens, indicating that these plasmids are associated with the virulence of *E. ictaluri* (Fernandez et al. 2001; Thune et al. 2007; Rogge et al. 2013). The urease associated genes are required for hydrolysed urea to produce ammonia, maintaining intracellular pH and/or providing a protective microenvironment during passage through or residence in the low-pH environment in the stomach (Booth et al. 2009). The identification of two mutants with insertions in urease-associated genes (*ureF* and *ureG*) suggests an important role for urease activity in *E. ictaluri* pathogenesis whether those mutants differed from the wild type in their virulence (Booth et al. 2009).

The role of type IV secretion systems (T4SS) (Christie & Vogel 2000; Cascales & Christie 2003; Juhas et al. 2008), and type VI (T6SS) (Zheng & Leung 2007; Silverman et al. 2012) in virulence, particularly with intracellular survival, was identified in Gram negative bacteria. Although the role of the T4SS, T6SS in pathogenesis of *E. ictaluri* has been confirmed, the proteins secreted and the pathogenesis mechanism of the *E. ictaluri* IV or VI secretion systems still remains unclear (Zheng & Leung 2007; Rogge & Thune 2011).

Table 1.2. Proteins encoded by genes of the type III secretion systems in *Edwardsiella ictaluri* (Rogge 2009; Park et al. 2012)

Abbreviation	Name	Putative function
EsaB	Putative TTSS apparatus protein B	Apparatus
EsaC	Putative TTSS apparatus protein C	Apparatus
EsaD	Putative TTSS apparatus protein D	Apparatus
EsaE	Putative TTSS apparatus protein E	Apparatus
EsaG	Putative TTSS apparatus protein G	Apparatus
EsaH	Putative TTSS apparatus protein H	Apparatus
EsaI	Putative TTSS apparatus protein I	Apparatus
EsaJ	Putative TTSS apparatus protein J	Apparatus
EsaK	Putative TTSS apparatus protein K	Apparatus
EsaL	Putative TTSS apparatus protein L	Apparatus
EsaM	Putative TTSS apparatus protein M	Apparatus
EsaN	Putative TTSS apparatus protein N	Apparatus
EsaO	Putative TTSS apparatus protein O	Apparatus
EsaP	Putative TTSS apparatus protein P	Apparatus
EsaQ	Putative TTSS apparatus protein Q	Apparatus
EsaR	Putative TTSS apparatus protein R	Apparatus
EsaS	Putative TTSS apparatus protein S	Apparatus
EsaT	Putative TTSS apparatus protein T	Apparatus
EsaU	Putative TTSS apparatus protein U	Apparatus
EsaV	Putative TTSS apparatus protein V	Apparatus
EsaW	Putative TTSS apparatus protein W	Apparatus
EscA	Putative TTSS chaperone protein A	Chaperone for translocon protein
EscB	Putative TTSS chaperone protein B	Chaperone for eseG
EscC	Putative TTSS chaperone protein C	Chaperone for translocon protein
EscD[**]	Putative TTSS chaperone protein D	Chaperone for eseI; encoded within plasmid pEI2
EseB	Putative TTSS effector protein B	Effector for translocon protein
EseC	Putative TTSS effector protein C	Effector for translocon protein
EseD	Putative TTSS effector protein D	Effector for translocon protein
EseE	Putative TTSS effector protein E	Effector for translocon chaperone (possible)
EseG	Putative TTSS effector protein G	Effector for translocon protein
EseH[*]	Putative TTSS effector protein H	Effector for translocon protein
EseI[**]	Putative TTSS effector protein I	Effector for translocon protein
Eser A	TTSS regulator protein A	Regulator for membrane sensor kinase
Eser B	TTSS regulator protein B	Regulator for cytosolic response
Eser C	TTSS regulator protein C	Regulator for AraC type transcriptional activator
Slt	TTSS regulator protein A	Soluble lytic transglycosylase

(*) gene of corresponding protein is not encoded by T3SS but by plasmid pEI1
(**) gene of corresponding protein is not encoded in T3SS but by plasmids pEI2

Up to the present (2014), only one study has been conducted to investigate the virulence factors in Vietnamese *E. ictaluri* strains. Rogge et al. (2013) identified the pathogenicity islands that encode the T3SS genes (*escD*, *esrC*, and *eseI*), the T6SS genes (*evpC*), and the urease enzyme amplified representative genes (*ureG*) in Vietnamese *E. ictaluri* isolates. However, the T4SS gene *virD4* was not observed in Vietnamese *E. ictaluri* isolates, suggesting that the Vietnamese isolates do not carry the *virD4* gene or possibly the entire T4SS locus. Rogge et al. (2013) also reported that Vietnamese *E. ictaluri* isolates from striped catfish were unable to replicate in head kidney derived macrophages or to cause mortality in USA channel catfish, indicating that Vietnamese isolates have reduced or no virulence in USA channel catfish. Further studies should be conducted to identify the proteins secreted by the *E. ictaluri* T3SS, T6SS as well as to confirm their role in pathogenesis of Vietnamese striped catfish *E. ictaluri* isolates.

1.1.6. Diagnosis

Classical fish pathogen identification is carried out by performing the following techniques: Phenotypic analysis based on microbial cell structure, cellular metabolism and differences in cell components followed by biochemical characterization.

Edwardsiella ictaluri can be isolated from infected organs using general culture media such as brain heart infusion agar (BHI) and tryptone soya agar (TSA) (Crumlish et al. 2002). Selective media for isolating bacteria belonging to the Enterobacteriaceae family such as Eosin-Methylene Blue (EMB) and *E. ictaluri* agar (EIA) (Shotts & Waltman 1990) were used for the recovery of *E. ictaluri* in infected striped catfish

(Crumlish et al. 2002). After 48 h incubation on TSA at 28°C, colonies of *E. ictaluri* appear off-white and translucent with an irregular surface and edge, and approximately 0.14 ± 0.03 mm in size (Crumlish et al. 2002). An *E. ictaluri* colony on EI agar is green and translucent, circular in shape with a dark yellow pinpoint centre resembling the appearance of a ''fried egg'' when compared directly with that of the discrete green colony of *E. tarda*.

As well as biochemical tests, serology tools have been developed for indirect detection of *E. ictaluri* infecting channel catfish (Saeed & Plumb 1987; Plumb & Vinitnantharat 1989; Bertolini et al. 1990; Lobb et al. 1993; Hassan et al. 2010). All six serological techniques consisting of bacterial agglutination, passive haemagglutination, complement-dependent passive haemolysis, indirect immunofluorescence, agar gel immunodiffusion or haemagglutination with fractions of immunized fish serum were sensitive to *E. ictaluri* lipopolysaccharide (LPS) antibody (Saeed & Plumb 1987).

Enzyme-linked immunosorbent assay (ELISA) has been applied to detect antibodies in channel catfish to *E. ictaluri* (Waterstrat et al. 1989). An ELISA assay has been developed as a reliable tool for the diagnosis and monitoring of *E. ictaluri* infection in channel catfish using an immune-dominant antigen of USA *E. ictaluri* (Klesius et al. 1991). An indirect fluorescent antibody (IFA) test has been used to diagnose *E. ictaluri* from artificially infected channel catfish fingerlings. The IFA test for detection of *E. ictaluri* in experimentally infected channel catfish compared with bacteriological culture given a 90.3% correlation between the 2 tests (Ainsworth et al. 1986).

Panangala et al. (2006) compared the IFA test with bacteriological cultured showed a 80.7% in sensitivity and a 83.9% in specificity. The study of Panangala et al. (2006) also showed the positive predictive value (PPV) (i.e. the conditional probability of disease) of the IFA test was generally high, while the negative predictive value (NPV) (i.e. the conditional probability of no disease) was relatively low. In the context of sensitivity and specificity, the results of these IFA tests were within the range generally considered efficient for simultaneous detection of *E. ictaluri* in outbreaks of disease in USA catfish systems (Greiner & Gardner 2000; Panangala et al. 2006a).

Polymerase chain reaction (PCR) is another diagnostic tool widely used in clinical diagnosis of *E. ictaluri* infection in catfish. A real-time PCR protocol targeting a transposon was developed to detect as few as two to three *E. ictaluri* cells from mixtures of non-infected catfish blood (Bilodeau et al. 2003). However, transposons are not considered good targets for PCR detection because they are mobile elements that might not be present in all *E. ictaluri* isolates. Sakai et al. (2009b) performed an analysis of upstream regions of the fimbrial gene cluster among strains of *E. ictaluri* and developed species-specific PCR methods for detection of *E. ictaluri*. The specificity and sensitivity of this PCR method was tested (Sakai et al. 2009b).

Loop-mediated isothermal amplification (LAMP) that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions is a sensitive strand displacement technique (Notomi et al. 2000). Several reports on LAMP-mediated diagnostic methods have been developed for bacterial pathogens associated with fish.

The first use of LAMP in aquaculture was reported for detection of *E. tarda* infecting Japanese flounder *Paralichthys olivaceus* (Savan et al. 2004)*.* A LAMP-based diagnostic system has been applied for detection of *E. ictaluri* from diseased channel catfish, *I. punctatus* by targeting a putative antigenic gene, *eip18* (Yeh et al. 2005)*.* This LAMP assay could detect *E. ictaluri* with a count of 20 colony forming units (cfu) from diseased channel catfish suggesting the LAMP assay can potentially be used for rapid diagnosis in hatcheries and ponds (Savan et al. 2005; Yeh et al. 2005). Advantage of this assay that it is simple and does not require sophisticated equipment, the LAMP assay is considered as a provisional method for preliminary screening and surveillance of *E. ictaluri* at farm (Yeh et al. 2005).

1.1.7. Treatment and prevention

Medicated feed containing antibiotics is one management approach applied in striped catfish farms to treat bacterial diseases generally and BNP in particular (Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). Enrofloxacin, amoxicillin or florfenicol have been recommended to treat BNP in striped catfish in Vietnam (Crumlish & Dung 2006). These antibiotics are the common drugs for treating bacterial disease in striped catfish farming in Vietnam (Phuong et al. 2005; Sarter et al. 2007; Ho et al. 2008; Rico et al. 2012). Enrofloxacin is known to act by inhibiting bacterial DNA gyrase (a type-II topoisomerase), thereby preventing DNA supercoiling and DNA synthesis (Serrano 2005; Cabello Filipe C. 2006; Managaki et al. 2007). Whilst the effect of florfenicol is to inhibit protein synthesis in bacteria, amoxicillin is believed to inhibit the synthesis of bacterial cell wall (Serrano 2005; Cabello 2006; Managaki et

al. 2007). However, antibiotic therapy should not be considered the best management practice since inappropriate use of antibiotics may generate resistance and high antibiotic resistance was shown in Vietnamese isolates to oxytetracycline, oxolinic acid and potentiated sulphonamides (Crumlish & Dung 2006; Sarter et al. 2007). Especially, the multi-resistance to 4 or 5 antibiotics consisting of amoxicillin, florfenicol, oxytetracycline, oxolinic acid and potentiated sulphonamides was observed in striped catfish *E. ictaluri* isolates indicating extensive or inappropriate use of these antibiotics in striped catfish farming in Vietnam (Sarter et al. 2007).

Vaccination is one of the best options to prevent infectious diseases. Vaccination has been studied and commonly applied in the USA channel catfish farming (Plumb & Vinitnantharat 1993; Thune et al. 1997; Klesius & Shoemaker 1999; LaFrentz et al. 2011). *E. ictaluri* is an intracellular pathogen then a modified-live bacteria vaccine may be more effective in inducing acquired immunity to protect channel catfish against *E. ictaluri* infection (Klesius and Shoemaker 1999; Thune et al. 1997). A modified-live *E. ictaluri* vaccine for immersion developed by Klesius and Shoemaker (1999) is licenced and marketed under the name AQUAVAC-ESC (LaFrentz et al. 2011).

Recently, vaccination of BNP in striped catfish has been applied in Vietnam. The first ever commercial fish vaccine (ALPHA JECT Panga 1) was licensed in 2013 and is now commercially available for Vietnamese farmers. ALPHA JECT Panga 1 is a vaccine for injection that provides protection against the bacterium *Edwardsiella ictaluri* causing disease in pangasius (Pharmaq 2013)

1.2. Environmental factors and *E. ictaluri* infectivity

Perturbation of the environment may increase the potential for disease in susceptible fish species (Snieszko 1974; Arkoosh et al. 1998). Several studies showed that outbreaks of *E. ictaluri* can occur when a susceptible host (e.g. channel catfish) encounters a virulent pathogen (*E. ictaluri*) under environmental conditions that are conducive to proliferation of the pathogen and stressful for the host (Hawke 1979; Waltman et al. 1985; Thune et al. 1993; Plumb 1999). This is a simplistic description for disease dynamics in aquaculture systems as many environmental variables are present and changing or interacting all the time. It is clear from the published literature that *E. ictaluri* infection in channel catfish occurs within a specific temperature range sometimes referred to as the "ESC window" (Areechon & Plumb 1983; Francis-Floyd et al. 1987; Baxa-Antonio et al. 1992; Mqolomba & Plumb 1992; Plumb & Shoemaker 1995; Hawke et al. 1998; Plumb 1999; Zheng et al. 2004). Channel catfish may be susceptible to infection at any water temperature, but a population is at greater risk in both natural and experimental outbreaks when water temperatures are in the range of 22°C to 28°C. Mortalities slow down and usually stop outside of this temperature range. Klesius (1992) speculated that the rapid change in water temperature from the immune-functional temperatures of 20-30°C to the immune-compromising temperatures of 11-17°C for channel catfish could be associated with increased horizontal transmission of *E. ictaluri* resulting in more ESC outbreaks. It is considered that both the non-specific and specific immune responses of fish are significantly decreased when fish are subjected to temperature stress or temperatures above the normal water temperature range of the fish (Ainsworth et al. 1991; Dexiang &

Ainsworth 1991; Mqolomba & Plumb 1992; Le Morvan et al. 1998). However, the severity of the disease may be further influenced by the rate of bacterial growth and expression of virulence factors, which can also be influenced by the environmental water temperatures (Waltman & Shotts 1986; Shotts et al. 1986; Mqolomba & Plumb 1992).

Dissolved oxygen might contribute to the infection *E. ictaluri* in channel catfish. Mqolomba & Plumb (1992) demonstrated that fish exposed to a low oxygen concentration (2.6 or 1.8 mg/l) had significantly higher *E. ictaluri* concentrations in all organs than those exposed to a near-normal concentration of dissolved oxygen (6.4 mg/l). This indicated that a low oxygen concentration that is not lethal by itself can cause higher *E. ictaluri* concentrations in tissues. Low dissolved oxygen concentration affected fish health and increased the infectivity of bacteria in fish (Walters & Plumb 1980).

It would appear from previous studies that salinity might be one of the major factors affecting the establishment of *E. ictaluri* infections in catfish (Plumb & Vinitnantharat 1989; Plumb & Shoemaker 1995; Park et al. 2012). The mortality of *E. ictaluri* infected channel catfish held in 0.3% NaCl was significant lower than those kept in 0% or 0.1% NaCl. However, a high concentration of NaCl (3%) was shown to induce hemagglutination activity which correlated with the expression of the fimbrial major subunit (*Fim A*) which is a 19.3 kDa protein leading to enhanced virulence of pathogens (Park et al. 2012).

pH of the environment has been considered a critical condition that increased the virulence of *E. ictaluri* in the head kidney derived macrophages of channel catfish (Booth et al. 2006; Thune et al. 2007; Rogge & Thune 2011). The survival and replication of *E. ictaluri* cultured in a low pH (pH<5) *in vitro* environment is significantly higher than those at neutral pH (pH= 7) (Booth et al. 2006; Thune et al. 2007; Rogge & Thune 2011). However, the link between pH and *E. ictaluri* susceptibility *in vivo* has not as yet been demonstrated. Further work is required to establish if pH is a true risk factor associated with *E. ictaluri* infections in catfish species.

Other husbandry and environmental conditions such as handling, close confinement (Walters & Plumb 1980; Li et al. 1993), improper diet (Tomasso et al. 1980; Li et al. 1993; Paripatananont & Lovell 1995; Wolters et al. 1996), poor water quality, and fluctuation of environmental factors (Walters & Plumb 1980; Baldwin & Newton 1993; Ciembor et al. 1995; Paripatananont & Lovell 1995; Wolters et al. 1996) all lead to increased susceptibility to infection in channel catfish. However, a single environmental variable such as water temperature can influence the disease progression affecting the host and the bacterium, which may depend on individual fish susceptibility (Ciembor et al. 1995). This may also contribute towards the variation in mortality rates reported during different clinical outbreaks.

Factors such as stocking density, fluctuating environment conditions and farm management variations, are commonly associated with intensive aquaculture practice and may contribute to the development of natural disease outbreaks of *E. ictaluri*

infections in catfish species (Thune et al. 1993; Hawke et al. 1998; Austin & Austin 2007). The study of Crumlish & Dung (2006) showed that no association could be found between BNP outbreaks and factors such as source and size of fry; stocking density (high stocking density did not always lead to BNP outbreaks), treatment after stocking time or feed type. This study was, however, performed in cage farmed *P. hypophthalmus* and as this is no longer commonly practised in Vietnam then further studies should be conducted to investigate the role of environment factors in infectivity in pond systems. This could include host susceptibility during the onset of *E. ictaluri* infection in striped catfish.

1.3. *Edwardsiella ictaluri* infection in striped catfish in Vietnam.

1.3.1. Striped catfish farming in Vietnam

Striped catfish *P. hypophthalmus* is one of the most common farmed species in Vietnam with production reaching 1.3 million tonnes in 2012 (MARD 2013). The popularity of striped catfish is due to its relatively fast growth rate, high flesh quality, tolerance to low oxygen and crowding, as well as its ability to adapt well to the various culture systems. It also presents an efficient feed conversion rate and accepts manufactured feed (Phuong & Oanh 2010; De Silva et al. 2010; De Silva & Phuong 2011).

Most striped catfish farming operations occur along the Mekong and Basin Rivers belonging to 10 provinces in Vietnam, an area of approximately 6000 ha in 2010 (Fig. 1.1) (Phan et al. 2009; Bui et al. 2010; De Silva et al. 2010; De Silva & Phuong 2011;

Khoi 2011; Da et al. 2013; MARD 2013). There are three production systems comprising of seed, nursery and grow-out systems (Phan et al. 2009; De Silva et al. 2010; De Silva & Phuong 2011).

Over the last decade the farming of striped catfish took precedence over than others farmed fresh water fish species such as tilapia, walking catfish, etc…, and pond farming became the most dominant production system in Vietnam (Khoi 2011). Small-scale farms of less than 1 hectare of water surface are still common (Halls & Johns 2013).
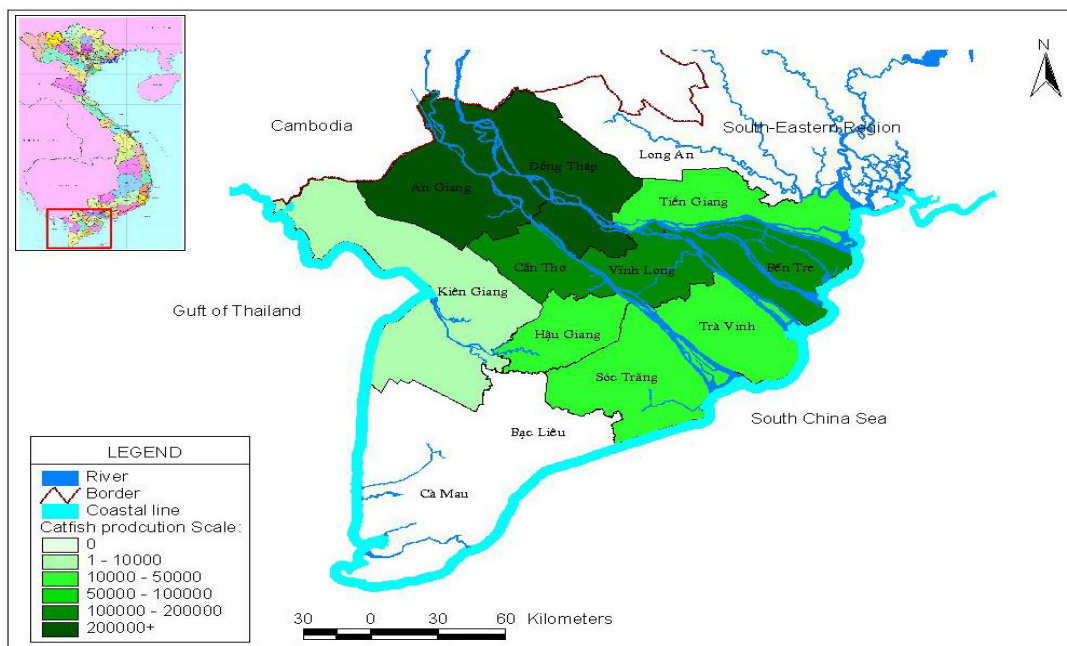
Figure 1.1. Striped catfish farming areas in Vietnam. Dark green shading indicates important provinces of production, light green shading indicates newly developed areas of production. (Source: Phan et al. (2011))

In Vietnam, hatchery-produced seed has now completely replaced the supply of seed from wild sources (Bui et al. 2010). There are more than 140 hatcheries in the Mekong Delta, mostly in An Giang and Dong Thap provinces (Phan et al. 2011) and they

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produced over 58 billion fry in 2008 (VIFED 2009; Sinh & Hien 2010; Halls & Johns 2013). Most hatcheries operated about 30 production cycles per year and most intensively between February and October (Halls & Johns 2013). Nursing is undertaken in small ponds, typically 1000-5000 m^2 and in shallow water (about 1.7 meters) for 3 months. Stocking density is normally from 200-400 fish per square meter (Bui et al. 2010; Halls & Johns 2013).

Fingerlings are stocked in the bigger ponds, typically 5000-10000 m^2. Pond depth typically ranges from 2.0 to 6.0 m with the great majority of farms having pond depths of between 3.5-4.5 m. At this stage, fingerlings are stocked the whole year round and stocking density is typically at 40 - 60 m^2 (Phan et al. 2009). Striped catfish attain a harvest weight of between 1.0-1.5 kg after 6 to 7 months depending on the size of fingerlings stocked (De Silva & Phuong 2011). In the period between 2002 to 2012, striped catfish has rapidly grown with an annual growth rate of 23.8%, from 151,000 tonnes to more than 1,285,500 tonnes with a total export value of approximately US$1.45 billion (Fig 1.2) and provides about 0.2 million jobs for local people in the Mekong Delta, Vietnam (Sinh & Hien 2010)
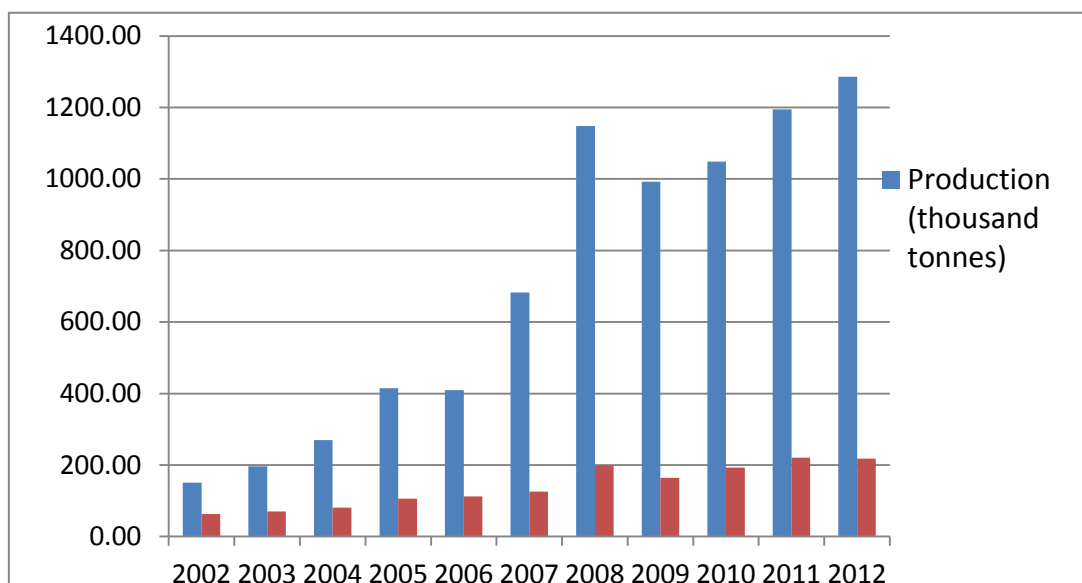
Figure 1.2. Production and yield of striped catfish in Vietnam from 2002-2012 (Source: MARD 2006; 2011; 2013)

1.3.2. *Edwardsiella ictaluri* infection in striped catfish in Vietnam

The main factor challenging the sustainability and profitability of the striped catfish farming sector in Vietnam is disease outbreaks (De Silva & Phuong 2011). Bacteria and parasites have been reported as common pathogens in striped catfish farming in Vietnam, all affecting the amount of fish produced (Crumlish & Dung 2006; Dung et al. 2008; Phan et al. 2009). Losses resulting from bacterial diseases have been estimated to be as high as 50% of total production compared with other diseases such as parasitic diseases or viral diseases (Phuong & Oanh 2010). Currently, BNP, Motile Aeromonad Septicaemia (MAS) and Jaundice disease are considered and documented as the most prevalent disease in striped catfish (Table 1.3) (Crumlish & Dung 2006; Phan et al. 2009; Ly et al. 2009; De Silva & Phuong 2011; Halls & Johns 2013; Luu 2013). Other diseases affecting the catfish industry are caused by the protozoan *Ichthyophthirius multifiliis*, and myxosporean infection.

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Table 1.3. Common diseases reported in striped catfish farming in Vietnam (Source: FAO 2010; Halls & Johns 2013; Luu 2013).

Disease	Agent	Type	Clinical signs
Bacillary Necrosis of *Pangasius* (BNP)	*Edwardsiella ictaluri*	Bacterium	Petechial haemorrhages on eyes and fin bases; white spots in the kidney, spleen and liver; some cellular necrosis
Motile Aeromonad Septicaemia (MAS)	*Aeromonas* spp. (mainly *A. hydrophila, A. sobria* and *A. caviae*)	Bacterium	Haemorrhages on eyes, body and fins; bloody ascites in the peritoneum, leading to swollen belly
Jaundice disease	Not confirmed	Not confirmed	Yellowing of the flesh

Bacillary necrosis of Pangasius (BNP) caused by *E. ictaluri* is considered to be the most serious disease occurring in catfish farms (Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; De Silva & Phuong 2011; Luu 2013). This disease has had an increasing impact over time and has been reported to cause 50-90% mortality of stocks during a single outbreak (Dung et al. 2004). In some cases, losses of up to 80% of fingerlings were reported in a single production cycle (Dung et al. 2004). The survey results of Khoi (2011) in 80 farms showed that the most frequent disease is BNP causing losses of 63.5%, followed by parasitic diseases and red spot disease accounting for 45.0% and 42.0% of fingerlings respectively. Moreover, according to the survey data of Luu (2013) in 46 farms located in four provinces (An Giang, Dong Thap, Vinh Long and Can Tho), 98% of farms reported fish losses due to BNP, followed by yellow discolouration (89%) and red spot/haemorrhage symptoms and/or swollen eyes (78%). Environmental conditions have also been thought to be associated with BNP, where it is suggested that *P. hypophthalmus* is seriously susceptible to *E. ictaluri* in June and July, which corresponds with the onset of the wet season and increased rainfall (Phuong et al. 2005; Phuong et al. 2007; Phan et al. 2009). Outbreaks of this disease have been

reported to be associated with stress-induced factors, including environmental degradation, omit stressors and low-quality stocked seed (Phuong et al. 2005; Phuong et al. 2007; Phuong & Oanh 2010). At the moment, BNP is widespread in Vietnam but effective treatment approaches are still limited. Although a commercial vaccine has been developed and will be applied in the near future for striped fish farming (MARD 2013), the use of injected vaccine is quite a difficult concept for many Vietnamese fish farmers moreover, the mortality of vaccinated fish of this vaccine is not significantly higher than non vaccinated fish (Pharmaq 2013) then antibiotics are still commonly used for the treatment of BNP. However, due to the rapid emergence of antibiotic resistance (Phuong et al. 2005; Sarter et al. 2007; Ho et al. 2008; Dung et al. 2008; Dung et al. 2009), antibiotic therapy is becoming less effective and more expensive both biologically and economically. The costs of treatments against fish disease, specifically BNP, increased from approximately 3% in 2004 (Crumlish & Dung 2006) to 5-5.5% of the production cost in 2010 (Khoi 2011). In addition, the study of Rogge et al. (2013) showed *E. ictaluri* to be fully resistant to flumequin, combined trimethoprim and sulfamethoxazole and highly resistant to streptomycin, chloramphenicol, and enrofloxacin suggesting inappropriate use of antibiotics in striped catfish farming in Vietnam. This is not only bad news for the reduction and treatment of BNP outbreaks but it could also enable other bacterial diseases to become established within the fish populations and production systems. The lack of information and knowledge of successful treatments is viewed as particularly problematic. Although BNP has greatly increased over the past few years in striped catfish farming in Vietnam, there has been limited information available on pathogenesis or epidemiological data as well as the

link to environmental factors and the infectivity of *E. ictaluri* in striped catfish at farm level. As a consequence, due to the lack of this information, the development of accurate treatment and effective vaccine use is still challenging.

1.4. Project aims

The main objective of this study was to investigate the connection of environmental factors with the infectivity of *E. ictaluri* in striped catfish (*P. hypophthalmus)*. The specific tasks involved were:

- Identification and characterisation of *E. ictaluri* with a range of laboratory tests including biochemical and molecular methods and antibiotic resistance. Fully understanding the role and behaviour of the pathogen *E. ictaluri* is important for developing prevention and control procedures, which will enhance profitability and production of striped catfish (chapter 2).

- Development of two reliable experimental fish challenge models including immersion and cohabitation for subsequent studies (chapter 3).

- Investigation of whether water pH and salinity are associated with the severity of *E. ictaluri* infection in striped catfish (chapter 4).

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CHAPTER 2. Identification of *Edwardsiella ictaluri* isolates recovered from natural infections in *Pangasianodon hypophthalmus* in Vietnam

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This chapter describes the biochemical, biophysical characteristics, plasmid profiles, and antibiotics resistance as well as the relatedness of *Edwardsiella ictaluri* isolates recovered in farmed striped catfish over last ten years from 2002 to 2011. All isolates between 2002 to 2009 used in this study were provided by Dr. Crumlish. The field studies for isolate collection in 2010 and 2011 were conducted by the main author, Nguyen Ngoc Phuoc over a period of three months each year. The isolates were collected both years at the same 40 farm sites consisting of 30 BNP outbreak farms and 10 recovered farms in 4 provinces of Vietnam. During these field studies, data on the farming practices and disease problems generally, and BNP outbreaks specifically were also collected by questionnaires and key informant interviews. This information was used to provide current information of BNP outbreaks in striped catfish as well as to establish the database of collected isolates, which were used in this study. The candidate organised the field trip and acquisition of isolates under the supervision of all co-authors. Part of the field work on sample collection was performed with technical assistance from Ms Wanna Siriminapong (IOA, UK).

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CHAPTER 2. IDENTIFICATION OF *Edwardsiella ictaluri* ISOLATES RECOVERED FROM NATURAL INFECTIONS IN *Pangasianodon hypophthalmus* IN VIETNAM

2.1. Abstract

In this study, 80 isolates of *E. ictaluri* were characterised using a variety of methods. All isolates were recovered from natural clinical disease outbreaks occurring in Vietnamese catfish farms from 2002 to 2011 located in 4 distinct geographical areas within Vietnam. The isolates were identified using both traditional primary and biochemical bacterial identification tests and their genetic relatedness was assessed using pulsed-field gel electrophoresis (PFGE). From these data, representative isolates from each genetic cluster were then selected for additional biophysical and biochemical investigation. The minimum inhibitory concentration (MIC) values were also investigated for these isolates. The PFGE results showed 6 main groups with a similarity of 82% and the corresponding genotypes of the prevalent isolates illustrated geographic and annual differences but no differences in biochemical characteristics were observed. Plasmid profiles identified 3 distinct groups. The results of MICs showed that all Vietnamese isolates were resistant to Oxolinic acid, Sulfadimethoxine /Ormetoprim (Romet), Oxytetracycline and Amoxicillin.

Keywords: *Edwardsiella ictaluri, Pangasianodon hypophthalmus*, biochemical characteristics, biophysical characteristics, PFGE.

2.2. Introduction

Edwardsiella ictaluri was identified as the aetiological agent of Bacillary Necrosis in Pangasius (BNP) (Crumlish et al. 2002) which continues to be the most serious infectious disease in farmed striped catfish in Vietnam. *Edwardsiella ictaluri* has been recognised as a significant pathogen of the USA channel catfish industry where outbreaks resulted in enteric septicaemia of catfish (ESC) (Hawke et al. 1981; Waltman et al. 1986). The bacterium is not only the causative agent of mortality in cultured ictalurids in the United States of America (USA) and pangasiids in Asia but has also been reported in danio *Danio devario* (Waltman et al. 1985), rainbow trout *Oncorhynchus mykiss* (Seçer et al. 2004), white perch (Pasnik et al. 2007), wild ayu *Plecoglossus altivelis* (Nagai et al. 2008; Sakai et al. 2008), Nile tilapia *Oreochromis niloticus* (Soto et al. 2012) and zebra fish *Danio rerio* (Hawke et al. 2013). The bacterium has been characterized and classified within the family *Enterobacteriaceae* (Hawke 1979; Hawke et al. 1981) and is described as a Gram-negative rod, cytochrome-oxidase negative and motile at 25°C (Hawke et al. 1981; Nagai et al. 2008; Ye et al. 2009) or non-motile at other temperatures (Waltman et al. 1986; Crumlish et al. 2002; Bartie et al. 2012). This bacterium is also described as having a high degree of biochemical homogeneity and being enzymatically non-reactive with urease, citrate, indole, protease, lipase and alginase (Hawke et al. 1981; Waltman et al. 1986). Varied molecular approaches have been applied to the USA *E. ictaluri* isolates to investigate their genetic relatedness. The results of these studies showed that there was a great degree of genetic homogeneity amongst these isolates from natural infection at different geographic locations over several years (Bertolini et al. 1990;

Cooper II et al. 1996; Panangala et al. 2005; Zhang & Arias 2007; Hassan et al. 2010; Yang et al. 2012). Moreover, *E. ictaluri* strains from Japanese ayu were antigenically homogeneous and closely related to two Vietnamese strains and three Indonesian strains from striped catfish *Pangasianodon hypophthalmus* and different from strains isolated from channel catfish *Ictalurus punctatus* in USA (Hassan et al. 2010). Recently, Bartie et al. (2012) reported the first molecular epidemiological study investigating the relatedness of the Vietnamese isolates to each other from 2002-2005. This study revealed that Vietnamese *E. ictaluri* isolates were a genetically distinct group compared with USA isolates. Study of characteristics and a genetically different within Vietnamese isolates will be useful for understanding the spread of *E. ictaluri* isolates in Vietnam and develop the efficiency treatment stratigies. The aims of this study were therefore to characterise new isolates which had been recovered from 2006 onwards and to compare these results with the existing data of Bartie et al. (2012).

2.3. Materials and methods

2.3.1. Bacterial isolate collection

Eighty isolates of bacteria from 80 farms which were presumptively identified as *E. ictaluri* recovered from clinical disease outbreaks in four provinces in Vietnam (Vinh Long, Can Tho, An Giang and Dong Thap province) from 2002 to 2011 were used in this study (Table 2.1). Fish from freshwater farms located in the four main catfish producing provinces of Vietnam were sampled during disease diagnosis visits. A total of 10 farms in 3 different districts of Vinh Long province, 15 farms in two districts of Can Tho city, 25 farms in four districts of An Giang province and 30 farms in 5 districts

of Dong Thap province were sampled (Figure 2.1). At each farm visit, four to six clinically moribund fish per pond were collected, examined grossly for any external or internal signs of disease and a full necropsy performed. Bacterial samples were aseptically taken at the farm site by inserting a sterile bacteriology loop directly into the kidney and spleen, and then streaked onto Tryptone Soy agar (TSA) (Oxoid, UK.) The inoculated agar plates were then sealed with nescofilm and incubated at 28°C and examined after 48h. Purity checks and single subcultures onto TSA were performed if required, where the dominant colony type was small, round, raised, opaque and off-white in colour. All isolates were then identified by biochemical tests and by PCR before storage in cryo-preservative in commercially prepared beads (Technical Service Consultant Ltd, UK) at -70°C.

Figure 2.1. Study area map (http://www.mekongdeltatours.com)
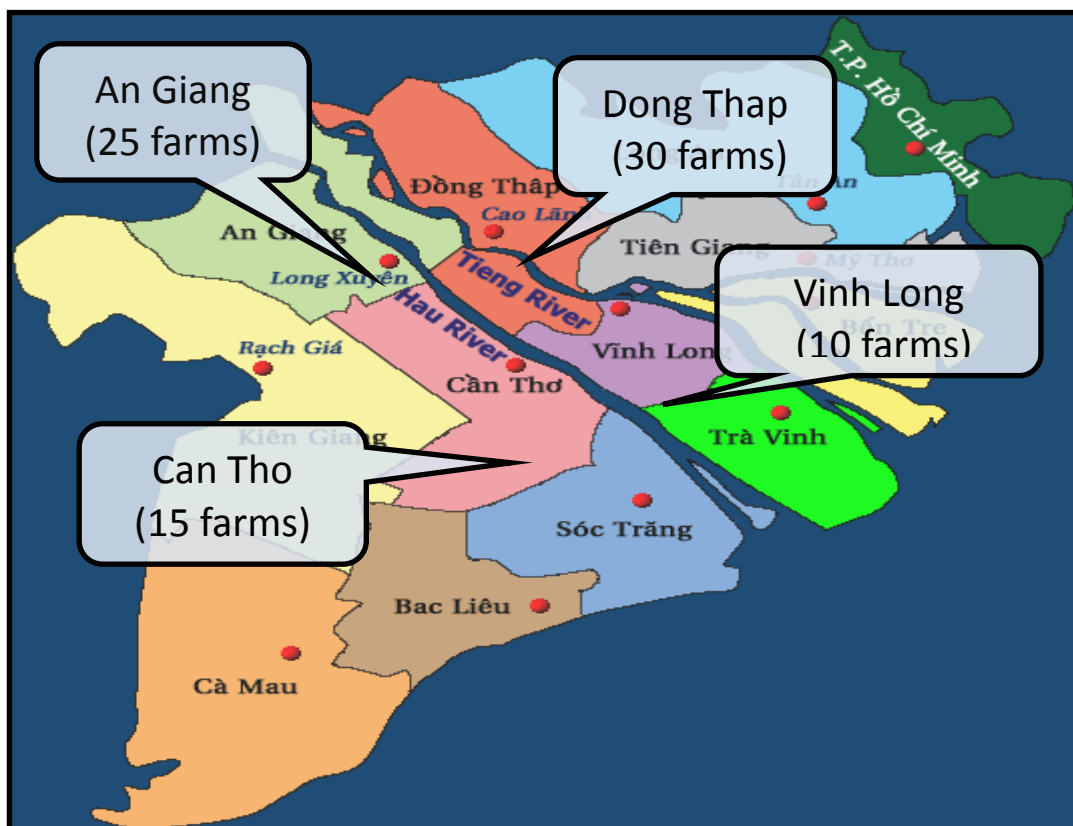
Table 2.1. List of isolates used in the study, accompanied with the year of isolation and province of origin

Year	Province & Isolate ID				Number isolates per year
	Dong Thap	An Giang	Can Tho	Vinh Long	
2002	001, 002. 003, 004	005, 006, 007, 024	008, 009	010	11
2003	011, 012, 013	014, 015	016	017	7
2004	020, 046	018, 019, 025	021		6
2005	026, 027, 029, 030, 039	038	028, 061	035, 036, 037	11
2006	043, 044, 023	031, 032, 034, 041, 042		040	9
2007	045, 047, 057, 022	048			5
2008	059, 060	049, 050	051, 058		6
2009	054, 055	053	052, 056		5
2010	065, 068	063, 064, 066	062, 067, 069		8
2011	075, 076, 080, 081	070, 071, 072	077	073, 074, 078, 079	12
Total	**31**	**25**	**14**	**10**	**80**

All isolates between 2002 to 2009 used in this study were provided by Dr. Crumlish (Institute of Aquaculture (IOA), University of Stirling). Each isolate represents a different disease outbreak.

2.3.2. Primary and biochemical identification tests

Traditional primary identification tests were performed following the methods described by Waltman et al. (1986) and conducted as described in Crumlish et al (2002). Unless otherwise stated the agar used was TSA (Oxoid UK), all chemicals were purchased from Oxoid or Sigma (UK) and assays were incubated at 28°C for 24-48h. Primary tests included Gram stain, oxidase, oxidation/fermentation (O/F), motility,

growth on Triple Sugar Iron (TSI) Agar and haemolysis when inoculated on 5% (v/v) sheep blood agar (BA). The motility test was performed by the wet-mount technique. The catalase reaction was determined following the method of Gagnon et al. (1959). The biochemical profiles of the isolates were determined using the commercially available kit API 20E (Biomerieux, UK) where the kit was used following the manufacturer's instruction except inoculated strips were incubated at 28°C and results read after 48h. From these primary and biochemical assays, the isolates could be identified as *E. ictaluri*. Additional biochemical tests were then performed to investigate fully the range of characteristics of the Vietnamese *E. ictaluri* isolates. These were implemented using a wide range of more traditional test tube methods: methyl Red, Voges-Proskauer, DNAse activity (Buller 2004), Lysine decarboxylase, Arginine decarboxylase, Ornithine decarboxylase (Crumlish 2011). Carbohydrate fermentative tests were carried out with purple broth base (Difco, UK) to which was added 5% of glucose, fructose, galactose, glycerol, maltose, mannose, or ribose. The ability of *E. ictaluri* to grow on selective agars was investigated by streaking a pure bacterial culture onto the following selective agars: *E. ictaluri* agar (EIA), MacConkey, Salmonella-Shigella, Brilliant green agar following the method of Shotts & Waltman (1990). These agars were purchased from Oxoid, UK.

For each test performed, a positive control *E. ictaluri* strain 12733 purchased from the National Collections of Industrial and Marine Bacteria (NCIMB, UK) was included. The results from the study of Waltman et al. (1986) for *E. ictaluri* recovered from ESC infected channel catfish in USA are included for comparative purposes.

2.3.3. Polymerase Chain Reaction (PCR) for identification of *Edwardsiella ictaluri*

A species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene for identification of *E. ictaluri* was performed following the methods of Sakai et al (2009b). All bacterial isolates were grown in 10 ml of sterile tryptone soya broth (TSB, Oxoid UK) at $28^{o}C$ for 24 hours prior to DNA recovery. A crude boiling extraction method (Queipo-Ortuño et al. 2008) was used where the bacterial suspension in Tris-Ethylenediaminetetraacetic acid (EDTA, Oxoid, UK) buffer (TE) (10mM Tris, 1mM EDTA) was heated at $95^{o}C$ for 10 minutes and then centrifuged at 13,000 rpm for 1 minute to remove cellular bacteria. Aliquots of the supernatant containing bacterial DNA was stored at $-20^{o}C$ until required. The primer set identified as ED_i, the forward primer (5'-CAGATGAGGGGATTTCACAG-3') and reverse primer (5'-CGCGCAATTAACATAGAGCC-3') were used to detect *E. ictaluri* (Sakai et al. 2009b). Ready-to-go-beads (Promega, UK), which contained freeze-dried DNTPs and Taq polymerase enzyme were used for the PCR following the manufacturer's instruction. The 25µl PCR reaction comprised of 1 PCR beads, 25 pmol of each primer (the forward and reverse of ED_i), 200ng of template DNA and nuclease-free water to volume. The thermocycling program consisted of an initial de-naturation step of 2 minutes at $94^{o}C$, 40 cycles of $94^{o}C$ for 30 seconds, $55^{o}C$ for 30 seconds, $72^{o}C$ for 1 minute and a final extension at $72^{o}C$ for 5 minutes. A negative control (purified DNA of *E. tarda* NCIMB 2034), a positive control (purified DNA of *E. ictaluri* NCIMB 13272) and an internal control (no DNA template) was included. The PCR amplification was conducted in Gene Amp PCR system 9700 (ABI, USA). The expected product was 470 bp (Sakai et al. 2009b).

2.3.4. Macrorestriction analysis by pulsed field gel electrophoresis (PFGE)

The PFGE followed the method of Bartie et al. (2012) with a slight modification. Briefly, 80 *Edwardsiella ictaluri* isolates were grown with vigorous shaking at 28°C for 24h before harvesting. The overnight suspension of 80 *E. ictaluri* isolates in Luria Broth (LB) (Difco, UK) were embedded with an equal volume of 2 % low melting point agarose (Flowgen, UK) and were lysed *in situ* overnight at 55°C in the lysis buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0, 1% Sarcosine) with 1mg ml^{-1} Proteinase K (Invitrogen, UK) added.

After that, slices of agarose containing the chromosomal DNA of 80 *E. ictaluri* isolates were digested overnight at 37°C in 150µl reaction containing 5 U of *Spe* I restriction enzyme (New England Biolabs NEB, UK), 1x Buffer 4 (NEB, UK) and 1x Bovine Serum Albumin (BSA) (NEB, UK). Then they were washed in 5 ml TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) six times for 30 min at room temperature. Finally, they were soaked in dilute TE at 4°C for 30 minutes before loading into the wells of 1% agarose gel (Gibco, UK) and 0.25 x Sodium Chloride - Tris - EDTA (TBE) running buffer (89nM Tris, 89mN boric acid (Oxoid, UK), 25nM EDTA pH 8.0).

The chromosomal DNA fragments were resolved into a pattern of discrete bands in the gel by a Rotophor apparatus (Biometra, UK) using the running program: switch time of 5-10 sec, run time of 25 hours, 120° constant angle, 200 V and rotor speed of 9.

The gel was stained with 0.5-1.0 µg ml^{-1} Ethidium bromide for 1h and de-stained in distilled water for 1h. The DNA restriction patterns of the isolates were analysed and

comparative analysis of their relatedness was performed using Gel Compare Software II (Applied Maths BVBA, Belgium).

2.3.5. Minimum Inhibitory Concentration (MIC) Assay

A total of 36 representative isolates covering all major PFGE groups identified were then selected based on the geographic location and time of collection, and their antibiotic sensitivity examined using Minimum Inhibitory Concentration (MIC) assay of 4 commonly used antibiotics in aquaculture (Table 2.2). The susceptibility of these isolates to Oxolinic acid (OA), Oxytetracycline (OT), Amoxicillin (AMO) and Sulfadimethoxine /Ormetoprim (Romet) were determined by MIC agar plate method using Iso-Sensitest Agar (ISA, Oxoid) as described in the study of Andrews (2001). These antibiotics were provided from Pharmaq, Norway. They were dissolved in appropriate solvents to make stock solutions and then diluted with distilled water according to the method of Ferraro et al. (2000) and ([CLSI] Clinical and Laboratory Standards Institute 2007).

All MICs were performed in triplicate with an antibiotic concentration ranging from 80 to 0.005µg ml^{-1}. *Aeromonas salmonicida* NCIMB 1102 and *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control standards. These strains were chosen for this assay on the basis of their susceptibility profile and their international acceptance for use in MIC assays. Additionally, they are representative of aquatic pathogens (Andrews 2001; Miller et al. 2003). The inoculum was prepared by adding 3-5 colonies of pure *E. ictaluri* to Iso-sensitest broth (ISB, Oxoid) and then incubating overnight at

28°C to achieve exponential growth, after which the bacterial suspension was adjusted by ISB to give an optical density (OD$_{600nm}$) value of 1. The viable colony counts were performed using the Miles and Misra method (Miles et al. 1938) and then 10-fold serial dilutions performed to give approximately 1 x 10^5 cfu ml^{-1} for the antibiotics test. The strains were inoculated on the antibiotic-containing ISA and antibiotic-free ISA (control) by a multipoint inoculator (Denley, England). Once the agar plates were inoculated they were incubated at 28°C and bacterial growth recorded after 48 hours. The MIC value was determined as the lowest concentration of antibiotic to inhibit visible bacterial growth. Interpretation was based on MIC (μg ml^{-1}) Interpretive Standard for Enterobacteriacae as recommended by the National Committee for Clinical Laboratory Standards ([CLSI] Clinical and Laboratory Standards Institute 2007).

2.3.6. Plasmid isolation

The plasmid profile of the 36 isolates (Table 2.2) previously selected for MIC was investigated following the method of Bartie et al. (2012). Briefly, *E. ictaluri* isolates were grown in LB media with vigorous shaking at 28°C for 24h before harvesting. The overnight bacterial suspension was centrifuged at 3,500 rpm and the cell pellet washed in STE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1M NaCl). Bacterial cells were lysed by chemical treatment in the presence of EDTA (50mM glucose, 25mM Tris and 10mM EDTA, pH 8.0 containing 10 μg ml^{-1} RNAase), the detergent containing 1% sodium dodecyl sulphate (SDS) and 0.2 N sodium hydroxide (NaOH). The alkaline lysate was neutralised with potassium acetate pH 4.8 which selectively precipitates out the protein and chromosomal DNA fraction. Cellular debris was removed by

centrifugation, leaving the re-annealed plasmid molecules within the supernatant fraction. The DNA was precipitated using isopropanol and the DNA pellets were re-suspended overnight in 100 ml of TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA) at $4^{o}C$ before running the samples using 0.7% agarose gel electrophoresis (Gibco) containing 0.2% ethidium bromide.

Escherichia coli V517 and *Escherichia coli* 39R861 purchased from NCIMB were used as reference plasmid markers. These isolates carry multiple plasmids of known size with *Escherichia coli* V517 containing 8 plasmids with the size of 2.0, 2.7, 3.0, 4.0, 5.1, 5.5, 7.2, and 54 kb and *Escherichia coli* 39R861 containing 4 plasmid with the size of 6.9, 36, 63, and 147 kb (Macrina et al. 1978).

Table 2.2. List of isolates, corresponding year of isolation and PFGE group used for MIC and plasmid profile study.

Year	PFGE Groups						Number strains per year
	A	B	C	D	E	F	
2002		001, 024	005, 008				4
2003		012, 013					2
2004	046, 020		018				2
2005		029, 038			037, 026	036	5
2006	044	042, 023				040	4
2007	021	034, 045		022			5
2008			049	059, 060			3
2009	055			054			2
2010	066, 063			062			3
2011	070, 071, 079		073, 074	076			6
Total	**10**	**10**	**6**	**6**	**2**	**2**	**36**

Thirty six isolates that used in the plasmid study represented from 36 outbreak diseases.

2.4. Results

2.4.1. Identification profiles

All of the Vietnamese isolates examined in this study were found to be Gram-negative rods, non-motile and cytochrome oxidase negative (Table 2.3). Results from Waltman et al. (1986) for *E. ictaluri* recovered from ESC infected channel catfish and *E. ictaluri* type strain NCIMB 12733 are included for comparative purposes (Table 2.3).

The differences in biochemical reactions among 80 isolates were observed on haemolysis and Glycerol tests. Only 10 of 80 isolates showed β-haemolysis on 5% sheep blood agar, and 5 of these 10 haemolytic isolates were negative in the glycerol reaction after 10 days (Table 2.3). Additionally, these 10 haemolytic isolates from 10 farms (outbreaks) belonged to group D after fingerprinting by PFGE and all 10 isolates were recovered from Dong Thap province only. The biochemical profile of the API 20E kit of Vietnamese isolates was only positive for Lysine decarboxylase (LDC) & GLU and gave an API 20E numerical profile of 4004000. All isolates were positive for Ornithine decarboxylase (ODC) when tested by traditional tube methods but these were negative using API 20E test kit. All isolates were positive for alkaline over acid reactions but 50/80 produced this without gas and 30/80 with gas and none of them produced H_2S on TSI.

Table 2.3. Biochemical characteristics of Vietnamese *Edwardsiella ictaluri* isolates used in this study. Results from Waltman et al. (1986) for USA *E. ictaluri* recovered from ESC infected channel catfish and *E. ictaluri* NCIMB 12733 are included for comparative purposes.

Characteristic or substrate tested	*E. ictaluri* (Waltman et al. 1986)	*E. ictaluri* NCIMB 12733	% Test strains	
			Positive	Negative
Gram	(-)	(-)		100
Motility	n/a	non		100
Oxidase	(-)	(-)		100
Oxidation, fermentation of glucose	+/+	+/+	100	
API 20E profile	n/a	4004000	4004000 [a]	
Lysine decarboxylase	Positive	Positive	100	
Arginine decarboxylase	(-)	(-)		100
Ornithine decarboxylase	(+)	(+)	100[b]	
Production of Methyl Red	(+) at 20°C & 37°C	(+) at 28°C	100	
Production of VP	(-) at 20°C & 37°C	(-) at 28^{0}C		100
Haemolysis	(+) at 20°C, 30°C & 37°C	(-) at 28°C	12.5	87.5
DNAse activity	(-)	(-)		100
Edwardsiella ictaluri agar	n/a	(+)	100	
MacConkey agar	(+)	(+)	100	
Salmonella-Shigella agar	(+)	(+)	100	
Brilliant green agar	(+)	(+)	100	
TSI	Alkaline/Acid without H$_2$S	Alkaline/Acid without H$_2$S	Alkaline/Acid without H$_2$S	
Produce gas on TSI			62.5	37.5
Growth in 1% NaCl	(+)	(+)	100	
Growth in 1.5% NaCl	(+)	(+)	100	
Growth in 2% NaCl	n/a	(-)	100	
Growth in 2.5% NaCl	n/a	(-)		100
Growth in 3% NaCl	(-)	(-)		100
Fructose	(+)	(+)	100	
D-galactose	(+)	(+)	100	
Glucose	(+)	(+)	100	
Glycerol	(+)	Weak (+)	92.5	7.25
Maltose	(+)	(+)	100	
D- mannose	(+)	(+)	100	
Ribose	(+)	(+)	100	

n/a: not applicable; (+): positive; (-): negative; [a] 100% of studied isolates gave the same results of API 20E; [b] 100% isolates showed positive for Ornithine decarboxylase (ODC) using tube methods but negative reaction with API 20E test kit.

2.4.2. PCR

All 80 Vietnamese isolates collected, plus the *E. ictaluri* NCIMB 12733 strain, produced by PCR an amplicon at the correct molecular weight (approximately 470 bp). All isolates were therefore confirmed as *E. ictaluri* (Figure 2.2).
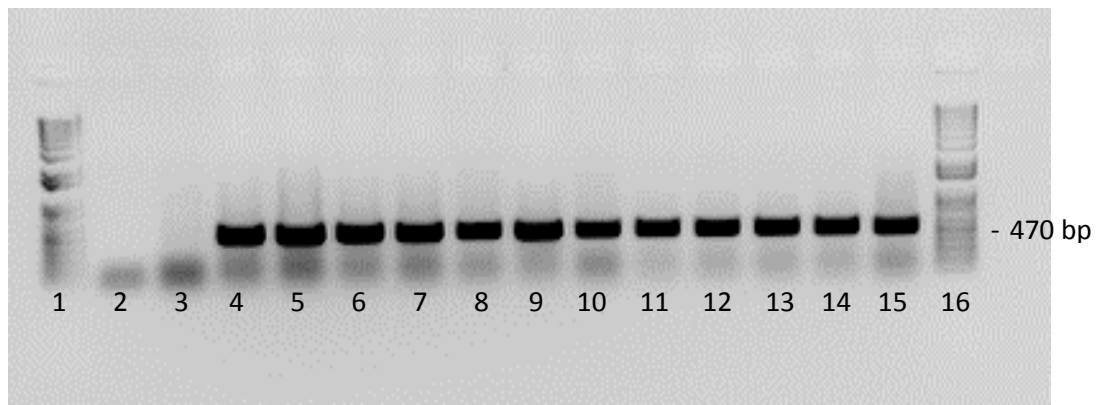
Figure 2.2. Example of PCR product from eleven *Edwardsiella ictaluri* isolates resolved by agarose gel electrophoresis showing Lane 1, 16: 1 kb ladder, lane 2: blank no DNA template, lane 3: *E. tarda* NCIMB 2034(negative control); Lane 4: E. *ictaluri* 12733 (positive control); lane 5 to 15: template prepared from *E. ictaluri* strain 001 to 010.

2.4.3. PFGE profile

The chromosomal DNA of *E. ictaluri* was digested into 10-13 restriction bands observed in the range of 48.5 to 436.5 kb by *Spe* I (Figure 2.3). The PFGE results of the 80 *E. ictaluri* isolates gave 6 distinct PFGE groups identified as A, B, C, D, E or F sharing 82% similarity. Isolates that belonged to 4 groups consisting of B (29 isolates), A (18 isolates), C (16 isolates), D (11 isolates) accounted for 93.8% of the total tested isolates.

Types A and B are the most common types. Isolates recovered from clinical BNP outbreaks in 2002-2007 in four provinces were predominantly in type A and B. Meanwhile the genotype C comprised isolates recovered from clinical outbreaks from 2002 to 2011 in four provinces. The genotype D (13.8%) consisted of isolates recovered from BNP outbreaks in the five-year period of 2007 to 2011 in Dong Thap only. Finally, the strains of group E (2.5%) and group F (3.8%) came from clinically affected fish recovered from 2005 to 2011 in Vinh Long and Can Tho province. Vietnamese isolates were distinct from the type strain NCIMB which was recovered from diseased channel catfish in USA.
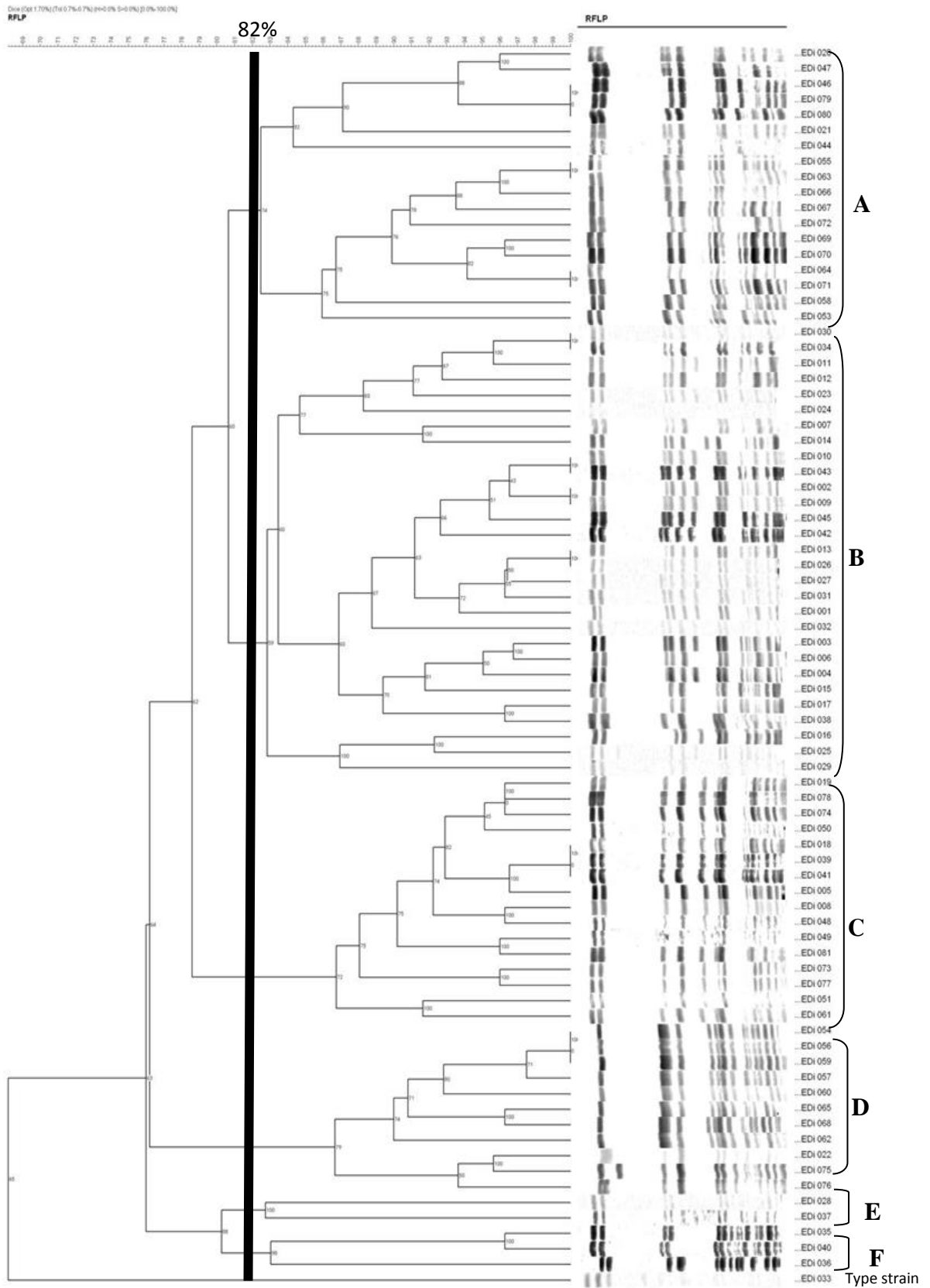
Figure 2.3. Chromosomal DNA restriction patterns of 80 isolates of *E. ictaluri.* Six PFGE groups are represented (cut-off 82% arbitrary)

2.4.4. Minimum Inhibitory Concentration (MIC) Assay

The results of MIC values of 4 antibiotics for 36 isolates of *E. ictaluri* are presented in Table 2.4. Briefly, all of the isolates had high MIC values for OA, which were greater than 80ug ml^{-1} (Table 2.4). This was similar for Romet, with the exception of 7 isolates that had a MIC at 5 ug ml^{-1} (Table 2.4). Again, higher MIC values were determined for most of the isolates (n=18) at 80ug/ml when tested against OTC but the range was more diverse for OTC and AMO (Table 2.4).

Table 2.4. Number of isolates that have the same MICs among 36 isolates testing for four antibiotics. Interpretive Standard as recommended by the National Committee for Clinical Laboratory Standards ([CLSI] Clinical and Laboratory Standards Institute 2007).

Concentration (µg ml^{-1})	Oxytetracycline (OTC)	Amoxicillin (AMO)	Oxolinic Acid (OA)	Romet
>80	18	13	36	29
80	3	0	0	0
40	1	1	0	0
20	1	1	0	0
10	1	0	0	0
5	0	2	0	7
2.5	1	16	0	0
1.25	9	0	0	0
0.6	2	3	0	0
0.3	0	0	0	0
0.16	0	0	0	0
0.08	0	0	0	0
0.04	0	0	0	0
0.02	0	0	0	0
0.01	0	0	0	0
0.005	0	0	0	0
MIC$_{50}$	80	2.5	>80	>80
MIC$_{90}$	>80	>80	>80	>80
% Resistance	70	40	ND[1]	ND[1]

ND: Not defined;

MIC values of the strains according to the PFGE groups with four antibiotics are presented in Table 2.5 where 100% of isolates belonging to group D, E were resistant

to all four antibiotics evaluated. On the other hand, 10 isolates accounting for 28% of test isolates which were sensitive to these antibiotics belonged to groups A, B and C (Table 2.5)

Table 2.5. Percentage of *Edwardsiella ictaluri* isolates belonging to each PFGE group (A to F) found to be resistant to four antimicrobial agents.

PFGE Group	Resistant percentage (%)			
	Oxytetracycline (OTC)	Amoxicillin (AMO)	Oxolinic Acid (OA)	Romet
A	40	30	100	20
B	50	20	100	30
C	67	67	100	17
D	100	100	100	100
E	100	100	100	100
F	100	100	100	50

2.4.5. Plasmid profile

All 36 isolates of *E. ictaluri* examined showed multiple plasmids (5 plasmids with the size ranging from 2.7 kb to 140 kb) (Table 2.6 and Figure 2.4).

Table 2.6. Characterisation of 3 different plasmid profile groups identified among 36 *E. ictaluri* isolates, including the number of plasmids, the size of the plasmids and the PFGE groups represented per plasmid profile group

Group	Number of isolates	Number of plasmids	Size of plasmid	PFGE groups
I	12	4	2.7 kb, 7kb, 35 kb and 140 kb	A, B, C, D, E, F
II	10	3	4kb, 7 kb and 140 kb	A, B, C, D, E, F
III	14	3	2.7kb, 4kb, 7kb	A, B

More than 60% of the *E. ictaluri* gave plasmid profiles 1 and 2 with the large molecular weight plasmid band of approximately 140 kb in size. The high molecular weight

plasmid band of about 35 kb was found in the plasmid profile group 1 only. In contrast,

profile group 3 contained multiple small molecular weight plasmid bands and plasmid

profile 3 came from isolates of PFGE group A and B only (Table 2.6).
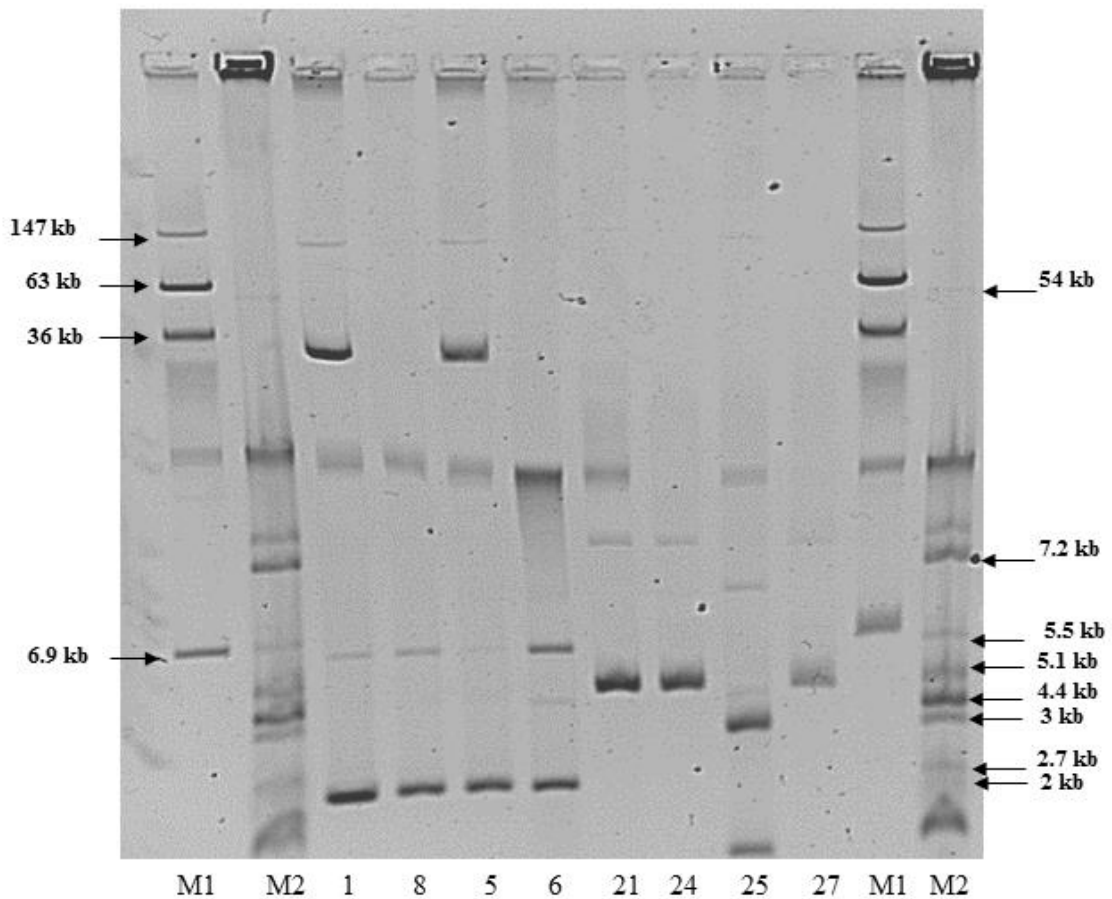
Figure 2.4.   Example of Electropherogram of plasmid DNAs from 8 *E. ictaluri* isolates with codification as isolates 1, 8, 5, 6, 21, 24, 25 and 27. Lane M1. *Escherichia coli* 39R861 (containing 4 known plasmids ranging from 6.9 to 147 kb), lane M2: *E. coli* V517 (containing 8 known plasmids ranging from 2 to 54 kb). The plasmid profile showed 3 distinct groups: (i) Profile I: 4 plasmids from 2.7 kb, 7 kb, 36 kb and 140 kb (including *E. ictaluri* isolates 1 and 5); (ii) Profile II: 3 plasmids from 4kb, 7 kb and 140 kb (consisting of isolates 21, 24, 25, 27); and (iii) Profile III: 3 plasmids from 2.7 kb, 4 kb, 7 kb (isolate 6,8)

2.5. Discussion

The results of this study showed high levels of phenotypic homogeneity between the *E. ictaluri* isolates investigated. This was similar to previous reports (Hawke et al. 1981; Hawke et al. 1981; Waltman et al. 1986; Plumb & Vinitnantharat 1989; Bader et al. 1998; Crumlish et al. 2002; Crumlish et al. 2010; Bartie et al. 2012) with a few exceptions in motility, haemolysin and glycerol fermentative reaction. In this study, *E. ictaluri* isolates from Vietnam were non-motile which was in agreement with previous studies (Waltman et al. 1986; Holt et al. 1994; Crumlish et al. 2002; Bartie et al. 2012). Recently, the study of Hawke et al. (2013) found that *E. ictaluri* isolates recovered from infected Zebra fish are very weakly motile at 28°C. Other studies described this bacterium as being motile at 20°C or 25°C but non-motile at 30°C or 37°C (Hawke et al. 1981; Plumb & Vinitnantharat 1989; Ferguson et al. 2001; Yuasa et al. 2003; Iwanowicz et al. 2006; Zhang & Arias 2007; Nagai et al. 2008; Ye et al. 2009; Liu et al. 2010; Geng et al. 2013). It is suggested the motility of this bacterium may vary depending on the incubation temperature.

Only 12.5% of Vietnamese isolates were haemolytic (β haemolysis) when inoculated on BA, while Waltman et al. (1986) reported that 97% of the USA channel catfish isolates were positive and elucidated that haemolysis was one of the most consistent characteristics of *E. ictaluri* when examined over a wide range of incubation temperatures. In some species of bacteria, haemolysin is an important virulence factor as the bacteria can acquire iron from lysed erythrocytes thus aiding in the penetration of mucosal layers, allowing intracellular survival, and assisting the spread

of the infection through the host (Williams & Lawrence 2005). However, there was no correlation between *in vitro* haemolysis activity and virulence of *E. ictaluri* (Williams et al. 2003; Williams & Lawrence 2005), although higher haemolysin activity was observed in virulent isolates compared with their attenuated isolates (Wong et al. 1998; May et al. 2000; Wang et al. 2003). The haemolytic activity of *E. ictaluri* was enhanced when isolates were adapted for growth in fish rather than *in vitro* growth condition (Stanley et al. 1994).

Studies of Williams et al. (2003) and Williams & Lawrence (2005) observed that 100% of their glycerol tests were negative, however in this study there were only 5 isolates that were positive for haemolysis that showed negative glycerol results. Most of the tested isolates had weak glycerol fermentation. The glycerol fermentation of *E. ictaluri* depends on the sensitivity of the basal medium used (Plumb & Vinitnantharat 1989). Additionally, there were some other variations in the glycerol test when a purple broth base with glycerol was used (Hawke et al. 1981; Waltman et al. 1986).

Biochemically, *E. ictaluri* has been described as ODC positive (Hawke et al. 1981; Plumb & Vinitnantharat 1989; Ferguson et al. 2001; Yuasa et al. 2003; Iwanowicz et al. 2006; Zhang & Arias 2007; Nagai et al. 2008; Ye et al. 2009; Liu et al. 2010; Geng et al. 2013). The Vietnamese isolates were positive for ornithine utilization when this was tested using the traditional decarboxylase assay. In the commercial API 20E kits, however, this was negative and in agreement with published literature (Bader et al. 1998; Crumlish et al. 2010; Hawke et al. 2013). This observation may be explained by

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the fact that the tube method is more sensitive than the commercial API 20E kit (Bader et al. 1998).

Based on the biochemical results, the primary and biochemical identification profiles of the culture collection used in this study appeared to be in general agreement with those in the public domain including published work on strains recovered from USA and Vietnam.

Results of the PFGE showed that there were dominant genotypes within the Vietnamese *E. ictaluri* detected from the different provinces. The genetic fingerprint of the Vietnamese isolates showed 6 distinct genotypes sharing 82% similarity that were identified from this bacterial culture collection for 10 year period. The type strain NCIMB belonged to a group distinct from the Vietnamese strains in agreement with the results of other studies of (Klesius et al. 2003; Hassan et al. 2010; Bartie et al. 2012). A previous amplified fragment length polymorphism (AFLP) genotyping study of Sakai et al. (2009a) revealed that genotype difference was observed depending on geographic location and year of isolation among *E. ictaluri* isolates from diseased ayu (*Plecoglossus altivelis*) from Japan, striped catfish (*Pangasianodon hypophthalmus*) from Indonesia, and channel catfish (*Ictalurus punctatus*) from USA. However, the recent PFGE genotyping study of Bartie et al. (2012) highlighted that two distinct genetic clusters sharing 64% similarity could be identified among *E. ictaluri* from diseased *P. hypophthalmus* from Vietnam during the period of 2001 to 2005, however no geographic difference was noted. The difference of the genotyping results between this study and the study of Bartie et al. (2012) reflected the difference in the

geographical origin and year of isolation of collection isolates used. The isolates used in the study of Bartie et al. (2012) were recovered from disease outbreaks in the five year period between 2001 and 2005 while the isolates in this study were recovered from 2006 onwards and some isolates from the study of Bartie et al. (2012) were included for comparative purposes. Importantly, only 1 isolate recovered from BNP outbreaks in Dong Thap in 2005 was examined in the study of Bartie et al. (2012) compared with 31 isolates from this province used in this study leading to genotype D consisting of isolates from Dong Thap province only. A wider geographic of isolate collection in this study gave 4 more genotype groups and noted the geographic genetic difference between Dong Thap, An Giang, Can Tho and Vinh Long provinces. The four provinces where the isolates were collected are the main striped catfish farmed areas in the Mekong Delta. The production of these provinces accounted for 74% of the national catfish production in 2010 (Phan et al. 2009; 2011). In addition, Dong Thap with 122 hatcheries, accounting for more than 70% of the total hatcheries in Vietnam was considered the main supplier of striped catfish fingerlings to the Mekong delta farms (Phan et al. 2011). The commercial activity of striped catfish fingerlings from Dong Thap to other provinces could be the main route of *E. ictaluri* transmission and lead to the genotype geographical difference between prevalent strains in four provinces. A phenomenon in terms of genotype geographical difference was found in the infection of *Streptococcus* in tilapia farming in China. Chen et al. (2012) considered that better adaptation or transmission of bacterial genotypes or a spread of the pathogen by commercial activity of tilapia fingerling movement, resulted in the genotype distribution of the pathogen.

Moreover, the genotype also varied in different years in the four provinces. Genotype D was found from disease outbreaks in 2007 to 2011, while genotypes A and B were defined by clinical outbreaks from 2002 to 2007. Genotypes E and F were only found between 2005 and 2007 after which these genotypes were not found in any isolates in BNP outbreaks. The finding of additional DNA polymorphism might be the result of evolution occurring within *E. ictaluri* species (Bartie et al. 2012) or just of wider temporal and spartial coverage. The genetic recombination and mutation process could lead to greater variation in genotype strains. These results have preliminarily suggested the possibility of genotypic difference on the basis of year of isolation. However, the number of isolates according to year of isolation in this study (Table 2.1) was not large enough to supply a sufficient diversity of isolates collection for concluding the annual trend. Further study on this issue is needed.

In terms of infectious disease outbreaks, *E. ictaluri* remains one of the most threatening diseases affecting striped catfish farming in Vietnam, and antimicrobial agents have been widely applied in the prevention and treatment of bacterial disease in aquaculture in Vietnam (Dung et al. 2008; Dung et al. 2009; Phan et al. 2009). However, since 2009 the Vietnamese Government controlled and limited the antibiotics used in aquaculture. Four common antibiotics, which are included in the 9 unrestricted chemicals available in Vietnam, were examined in this study (MARD 2009). The results of the antibiotic susceptibility showed that Vietnamese *E. ictaluri* isolates examined had multiple antibiotic resistance and this occurred amongst all isolates in the four provinces over a ten year period. Antibiotic susceptibility revealed

Oxytetracycline resistance among the *E. iclaturi* isolates in agreement with previous studies from the same region (Crumlish et al. 2002; Dung et al. 2008; Bartie et al. 2012).

The Vietnamese *E. ictaluri* isolates showed a higher level of resistance to Romet than the USA *E. ictaluri* isolates. In this study, only 18% of Vietnamese isolates compared with 100% of USA isolates in the study of Waltman & Shotts (1986) were sensitive to this antibiotic. Recently, Hawke et al. (2013) confirmed that the USA Zebra fish isolates of *E. ictaluri* were susceptible to the antibiotic Romet.

All the tested isolates in this study were basically resistant to the quinolone oxolinic acid as shown from the MIC results. Previous studies (Waltman & Shotts 1986; Stock & Wiedemann 2001; Dung et al. 2008) have shown full susceptibility of *E. ictaluri* to amoxicillin while the MIC results in this study had high levels of resistance (40% tested isolates) to this antibiotic.

Oxytetracycline and Sulfonamides have been used commonly in the treatment of *E. ictaluri* infection in USA because there are restrictions as to which antibiotics are licensed for use in each country and importantly no evidence of resistance of *E. ictaluri* to these antibiotics was noted (Waltman & Shotts 1986). Recently, the study of Hawke et al. (2013) highlighted that *E. ictaluri* isolates from zebra fish in USA were susceptible to oxytetracycline, florfenicol and enrofloxacin. However in Vietnam *E. ictaluri* showed either partial or full resistance to these antibiotics (Crumlish et al. 2002; Ho et al. 2008; Dung et al. 2008). It suggests that isoaltes become more resistant to these antibiotic

over past decade. The use of isolates that were sampled from 2006 onwards in this study led to the MICs of these isolates to 4 antibiotics being higher than reported in previous studies (Crumlish et al. 2002; Ho et al. 2008; Dung et al. 2008). Futhermore, 100% of isolates belonging to genotype D, which was recovered from Dong Thap province, were resistant to all four antibiotics. Moreover, multiple antibiotic resistances to all four antibiotics were also observed in isolates of genotypes A, B, E and F recovered from Vinh Long, Can Tho, and An Giang (Table 2.5). It is perhaps readily explained because these four antibiotics have been used to treat a variety of striped catfish diseases, including BNP outbreaks, over many years. This also indicates the effect of the wide and non-controlled application of antibiotics in the treatment of bacterial disease in striped catfish leading to the high resistance of bacteria to antibiotics (Alderman & Hastings 1998; Dung et al. 2008).

Lobb et al. (1993) suggested that plasmid analyses can be used to determine whether structurally distinct strains of *E. ictaluri* are emerging in major channel catfish aquaculture areas. In this study, the plasmid profile of 36 isolates of *E. ictaluri* examined showed that the multiple plasmids belonged to 3 groups but the correlation of plasmid profile groups with the PFGE groups was not clear. Some isolates with the same PFGE pulsotype had different plasmid profiles (Table 2.6). It may be that there is quick adaptation (for example antibiotic resistance development) through acquisition of plasmids among isolates (Newton et al. 1988; Fernandez et al. 2001; Schmidt et al. 2001).

Generally, the results of plasmid profiles of 36 isolates examined did not show any trend of geographical region or year of isolation. This might be because the number of isolates of each PFGE group used in this study was not sufficient to evaluate any trends. Plasmid profile III containing small molecular weight plasmids was observed in isolates recovered from 2001 to 2006. On the other hand, isolates that belonged to plasmid profiles I and II contained a 140 kb plasmid which was found distributed among the isolates recovered over the 10 year period in all four provinces. These results were in agreement with plasmid profiles described in previous studies (Dung et al. 2008; Dung et al. 2009; Bartie et al. 2012). A high molecular weight *tetA*-carrying plasmid of the trans-conjugant (140 kb) belongs to the *incK* group showing resistance to tetracycline, trimethoprim and sulphonamides (Dung et al. 2009). The multidrug resistant plasmids are widely distributed among enterobacterial isolates from agriculture sources (Fricke et al. 2009; Welch et al. 2009). A similar plasmid belonging to the *incA/C*, namely pRA1 of approximately 144 kb is a transferable antibiotic resistance plasmid conferring resistance against sulfonamides and tetracyclines found in the fish pathogen *Aeromonas hydrophila* (Aoki et al. 1971; Fricke et al. 2009). By comparing the genome of the *incA/C* plasmid pRA1 and of the cryptic *incA/C* plasmid pRAX isolated from *Escherichia coli* trans-conjugant D7-3, Fricke et al. (2009) also stated that bacteria are able to share a plasmid backbone in conjugative transfer operons for horizontal propagation but are stable in the host chromosome during vegetative growth.

Resistance transfer correlated with a 150 kb plasmid suggesting the presence of a multidrug resistance plasmid was also found in the USA *E. ictaluri* isolates by Welch et al. (2009). These authors also emphasized that a high molecular weight plasmid conferred an increase in the resistance of *E. ictaluri* to all three antimicrobial drugs that are currently approved for use in aquaculture in the United States (florfenicol, ormetoprim-sulfadimethoxine, and oxytetracycline). The presence of this plasmid could affect the performance of therapeutic antimicrobial drugs in U.S. aquaculture. This may help to explain the failure of antibiotic treatment in Vietnamese catfish farming in recent years. The presence of antibiotic resistant plasmids in *E. ictaluri* isolates will may affect drug sensitivity and combined with a lack of a therapeutic regime and non-registration of therapeutic treatment by farmers are the most likely reasons that the antibiotic treatments have not been successful in Vietnam.

From the plasmid profile generated, plasmids of approximately 4 kb and 35 kb were found in isolates sampled in all four provinces, in agreement with a previous study by Bartie et al. (2012). By contrast, a smaller plasmid of approximately 2.7kb was not mentioned in any previous plasmid studies investigating Vietnamese *E. ictaluri* isolates. Furthermore, a larger plasmid of approximately 7kb identified in this study was different to the documented 10 kb plasmid in the previous study of Bartie et al. (2012). However, the role of these plasmids has not been described and it needs to be further studied.

The results from this study confirmed that the Vietnamese *E. ictaluri* isolates are generally homogeneous and very similar to other *E. ictaluri* isolates described from natural infections in other fish species. Subtle differences were found during the molecular epidemiology study with the addition of 3 PFGE groups and smaller and mid-range plasmids. These had not been previously recognised in Vietnamese or USA strains and it is likely that further work is required to elucidate their significance in pathogenesis of BNP infections. This is a worry for subsequent treatments and it is likely that a combination of antimicrobial resistance development combined with a lack of rigorous therapeutic regimes in Vietnam have contributed to this finding. However, further study should be done to confirm the plasmid profiles in Vietnamese *E. ictaluri* isolates. Better understanding of the context of plasmid profile and a genetically distinct group among Vietnamese *E. ictaluri* isolates might help to develop better treatment and prevention strategies in striped catfish farming in Vietnam.

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CHAPTER 3. Experimental challenge model of *Edwardsiella ictaluri* in striped catfish *Pangasianodon hypophthalmus* (Sauvage)

Nguyen Ngoc Phuoc, Niall Auchinachie, Randolph Richards, Mags Crumlish

This chapter presents two challenge models of experimental *Edwardsiella ictaluri* infection in striped catfish. The main author, Nguyen Ngoc Phuoc, designed the study and conducted the analytical work under the supervision of all co-authors. Part of the challenge work on sample collection was performed with technical assistance from Mr. Niall Auchinachie (IOA, UK).

The text of this chapter is presented as a publication-ready manuscript. This manuscript was an oral presentation at the World Aquaculture Conference 2012, Prague, Czech Republic, and a poster presentation at the International Symposium on Diseases in Asian Aquaculture VIII, Mangalore, India (November, 2011), and at the Symposium on Microbial Management in Ghent University, Belgium (August, 2012).

CHAPTER 3. EXPERIMENTAL CHALLENGE MODEL OF *Edwardsiella ictaluri* IN STRIPED CATFISH *Pangasianodon hypophthalmus* (Sauvage)

3.1. Abstract

A reproducible bacterial immersion challenge model was developed and performed *in vivo*. All experiments were conducted using a bacterial isolate of *Edwardsiella ictaluri* that had been recovered during a natural outbreak of BNP in farmed striped catfish *Pangasianodon hypophthalmus* in Vietnam. Fish were exposed to different bacterial concentrations by immersion for up to 30 seconds, resulting in a cumulative percentage mortality of 63%. Three to 4 days post-bacterial challenge, fish showed gross clinical signs of disease commonly associated with *E. ictaluri* infection in *P. hypophthalmus* and *E. ictaluri* was recovered and identified from these fish. No mortalities or morbidity were observed in the control fish groups and no clinical signs of disease or bacteria were recovered from these fish. This immersion challenge model has been repeated several times using different strains of *E. ictaluri* with similar outcomes. Hence, this study provides data on a reliable and robust experimental challenge model suitable for use in further studies. Moreover, a cohabitation challenge was evaluated and found to be an alternative challenge method, although the mortalities among the infected fish were lower at around 15-40%. Additionally, this study confirmed the horizontal transmission of *E. ictaluri* in striped catfish and elucidated that cohabitation challenge could be used in reproducing the disease under controlled conditions.

Keywords: *Pangasianodon hypophthalmus, Edwardsiella ictaluri,* Bacillary Necrosis of *Pangasianodon*, immersion challenge, cohabitation challenge.

3.2. Introduction

Bacillary necrosis of Pangasianodon (BNP), one of the most serious diseases of striped catfish in Vietnam was first described in 2001 (Ferguson et al. 2001) where Crumlish et al. (2002) identified *E. ictaluri* as the causative agent of natural outbreaks of BNP and confirmed aetiology through experimental studies (Crumlish et al. 2010). Similar to Enteric Septicemia of Catfish (ESC) in the USA channel catfish industry, outbreaks of BNP within the Vietnamese *P. hypophthalmus* industry have caused significant economic damage through fish losses resulting in decreased production and further financial losses through increased application of antibiotic treatments (Dung et al. 2008; Thinh et al. 2009). During a natural outbreak of *E. ictaluri,* 50-90% mortality has been reported on the affected farms in Vietnam (Dung et al. 2004). The use of chemical therapy and vaccination in treatment and prevention of *E. ictaluri* in striped catfish were considered (Dung et al. 2008; Thinh et al. 2009) and work is on-going for the first commercially approved fish vaccine in Vietnam (Pharmaq, 2013).

At present experimental challenge models have been used to understand pathogenesis and evaluate treatment and prevention strategies. Several studies have applied challenge methods with *E. ictaluri* (Newton et al. 1989; Plumb & Vinitnantharat 1993; Stanley et al. 1994; Plumb & Shoemaker 1995; Ciembor et al. 1995; Plumb & Shoemaker 1995; Ciembor et al. 1995; Klesius & Shoemaker 1999; Williams et al. 2003; Iwanowicz et al. 2006; Pasnik et al. 2007; Thinh et al. 2009; Ye et al. 2009; Crumlish et al. 2010) for varied reasons. However, a standardised approach has not yet been produced for this bacterium in striped catfish.

Generally, performing *in vivo* bacterial challenge studies under experimental conditions is difficult to standardise in terms of repeatability. Production of a reliable challenge model in fish is not a simple task and many variables must be considered and investigated. Pathogen exposure methods can include injection, oral, hyperosmotic immersion, direct immersion, and cohabitation (Bell et al. 1984; Elliott et al. 1991), but injection is the most widely adopted method used in aquaculture. Each exposure method has its own benefits and drawbacks. Pathogen exposure through injection has been criticised as this allows the bacterium to bypass the protective function of the skin mucous layer, allowing considerably faster development of the disease than during natural disease progression (Amend 1980; Nordmo & Ramstad 1997). However, injection challenge also imposes the additional stress of handling each individual fish (Murray et al. 1992). Immersion (McCarthy et al. 1984) and cohabitation studies (Bell et al. 1984) have shown promise as aquatic exposure methods and they represent a more natural route of pathogen entry than injection however, these methods are more difficult to control and standardise (Nordmo & Ramstad 1997). Importantly, the mortality percentage of 60% to 80% of the population should be achieved in the challenge treatment. This value is assumed to be an adequate level of mortality in order to give a sufficient number of infected fish in the group for evaluation of the significance of an experimentally applied infection (Amend 1981). Some challenge studies were conducted by intraperitoneal (i.p.) injection or immersion to confirm the infection of *E. ictaluri* in Vietnamese striped catfish. Previous studies (Thinh et al. 2009; Crumlish et al. 2010) have conducted immersion challenge, but although these studies successfully reproduced

clinical signs of disease in the exposed fish, the challenge dose and the cumulative mortality varied. Immersion exposure of the fish to the pathogen reflects a natural route of infection and is considered a best challenge model particularly when using the pathogen challenge study to evaluate preventative treatments including vaccines (Nordmo & Ramstad 1997; Aoki et al. 2005). Cohabitation has been considered as the model which most closely resembles the natural infection but it is difficult to achieve and even more problematic to standardise than immersion challenge (Nordmo & Ramstad 1997). Co-habitation challenge study helps to identify the horizontal transmission of the pathogen amongst the fish population. Horizontal transmission has been observed in channel catfish (Shotts et al. 1986; Klesius 1994; Gaunt et al. 2006), however, the mechanism of horizontal transmission of the bacterium in striped catfish is still not well known. The aim of this study was to provide a reliable and reproducible immersion challenge model of *E. ictaluri* infection in striped catfish with a lethal concentration affecting 60% of the exposed population (LC_{60}). A successful model would then be applied in subsequent studies.

3.3. Materials and Methods

3.3.1. Fish

The fish used for the experimental studies were obtained from a stock population held in the Aquaculture Research Facility (ARF), University of Stirling. These fish were originally purchased from a farm in central Thailand and had been health certificated for disease free prior to use. The stock fish were maintained in 200L fibre glass tanks at $28^{o}C \pm 2^{o}C$, and fed a commercial salmonid diet. In total for the challenge studies,

84

10 fish per treatment group were allocated to 100 litre tanks. The fish were starved for 24h prior to removal from stock tanks and they received the bacterial challenge on the same day of movement. An acclimation period was not required for these fish, as they had come from the stock tanks in the same aquarium using the same single water source. The weight of the fish used for the bacterial challenge studies was 15 ± 2 g.

3.3.2. Bacterial strain

A bacterial strain of *E. ictaluri* recovered from a natural outbreak of BNP in Vietnamese *P. hypophthalmus* was used for all challenge studies. This isolate was identified as *E. ictaluri* by Gram stain, oxidase, O/F, motility; growth on Triple Sugar Iron (TSI) Agar (Oxoid, England) and haemolysis on 5% sheep blood agar (performed at 28°C) as described by Crumlish et al. (2002; 2010). The biochemical profiles of the isolates were determined using the commercial kit API 20E, purchased from Biomerieux (UK) where the kit was used following the manufacturer's instructions except for incubation at 28°C and reading after 48h.

A species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene for identification of *E. ictaluri* was performed following the methods of Sakai et al (2009) as previously described in 2.2.3 chapter 2.

3.3.3. Bacterial challenge studies

Prior to performing the challenge studies, the *E. ictaluri* strain was passed through naive fish twice as per standard practice at the Institute of Aquaculture (IOA),

University of Stirling, United Kingdom following prolonged storage of the bacterial strain. The bacterial suspension was grown as previously described, centrifuged and re-suspended in sterile 0.85% saline to give a high bacterial concentration. One hundred microliters of this was then injected by i.p. into the fish and recovered from moribund/dead fish directly onto TSA from the spleen and/or kidney. This was repeated twice and the identification of the isolate (identified as ex-passage 2) recovered from the fish was confirmed as described above and then used for the challenge studies.

The challenge inoculum was produced by adding 3-5 colonies of pure *E. ictaluri* isolate ex-passage 2 grown on Tryptone Soya Agar (TSA, Oxoid England) into 50 ml of sterile TSB (Oxoid, England). This was then incubated to mid logarithmic phase (140 rpm, 28°C) in a shaking incubator (Kuhner shaker, ISF-1-W, Switzerland). After 24h the bacterial broth suspension was centrifuged at 3,500 rpm (Sanyo NSE Mistral 2000R, Japan) and the cell pellet re-suspended and adjusted to give an optical density (OD$_{600nm}$) value of 1 using 0.85% sterile saline. The viable colony counts were performed using the Miles and Misra method (Miles et al. 1938) and then 10-fold serial dilutions performed to give approximately 1×10^7 cfu ml^{-1} for the challenge studies.

An initial bacterial challenge experiment was conducted using 5 groups of 10 fish per group. All fish were exposed to a single concentration of 1.08×10^7 cfu ml^{-1} for various exposure times of 1, 2, 5, 10, and 15 minutes (Table 3.1). The control fish group was

treated in the same way except they were exposed to sterile 0.85% saline solution for the longest exposure time of 30 minutes. Immersion doses were chosen based on a series of pre-challenge studies performed previously at the Aquaculture Research Facility (ARF), University of Stirling (data not shown).

Table 3.1. The concentration of *E. ictaluri*, exposure time and number of fish per treatment group that were used in an initial challenge experiment.

Treatment group	No. Fish	Immersion time	Bacterial concentration
1	10	30 min	
2	10	15 min	
3	10	10 min	1.08×10^7 cfu ml^{-1}
4	10	5 min	
5	10	2 min	
6	10	1 min	
Control	10	30 min	0.85 % sterile saline water

3.3.4. Challenge experimental design

A second immersion challenge was performed which was a refinement of exposure time from the first challenge (Table 3.2).

The second study had 4 treatment groups with 10 fish per group exposed to a single concentration of bacteria (1.06×10^7 cfu ml^{-1}) for 30 seconds, 1 minute or 2 minutes. In this study, there were triplicate tanks per treatment group except the control treatment which was only a single replicate exposed to sterile saline for 2 minutes (Table 3.2).

Table 3.2. Challenge experimental design demonstrating the concentration of *E. ictaluri,* exposure time, number of fish and replicates per treatment group.

Treatment group	No. Fish	Bacterial concentration	Exposure time	No. replicates
1	10	1.06×10^7 cfu ml^{-1}	30 sec	3
2	10	1.06×10^7 cfu ml^{-1}	1 min	3
3	10	1.06×10^7 cfu ml^{-1}	2 min	3
Control	10	0.85% sterilized saline water	2 min	1

3.3.5. Cohabitation experimental design

Co-habitation studies were performed using varied routes of bacterial exposure in the 'seed' fish. All seed fish were identified by removing the adipose fin, which had previously been shown to have no adverse effects on the experimental challenge (Crumlish pers. com.). Once the seed fish had received the bacteria they were then kept with the un-exposed animals in the same tank. This was described as direct contact co-habitation. The experimental studies and designs are described in Table 3.3. An appropriate control was used for each co-habitation treatment group where the seed fish was identified by fin-clip prior to being held in sterile saline water for 15 min or receiving 0.1ml of sterile saline by i.p. injection and then placed into the same tank with the other fish in the treatment group. Each treatment and control group consisted of 1 seed fish and 9 apparently normal fish per tank and was performed in duplicate (Table 3.3).

Table 3.3. Experimental design for the direct contact cohabitation challenge according to the concentration of *E. ictaluri*, the method of experimental infection in seed fish, number of uninfected fish per treatment group.

Treatment group	Number of uninfected fish	Number of seed fish	Infected route	Bacterial concentration
1	9	1	i.p. injection	1.1×10^6 cfu fish^{-1}
2	9	1	i.p. injection	0.1 ml of 0.85 % sterile saline water
3	9	1	immersion	1.1×10^7 cfu ml^{-1} for 15 minutes
4	9	1	immersion	0.85 % sterile saline water for 15 minutes

For each bacterial challenge study the water temperature was $26 \pm 2^{o}C$ and the duration of the study was 15 days. All fish were provided with aeration through an air stone and were fed a maintenance ration twice daily. The water temperature and mortality/morbidity was checked and recorded 4 times per day.

Moribund and freshly dead fish were necropsied and examined grossly for any external and internal clinical signs of disease. Bacterial samples were taken aseptically from the kidney and spleen by inserting a sterile bacteriology loop directly into the organs and then streaked onto TSA plates, which were sealed and incubated at $28^{o}C$. These were checked for bacterial growth and purity. If required they were sub-cultured and isolates morphologically similar to *E. ictaluri* were identified as described in chapter 2. Liver, spleen, kidney, brain, gill and digestive tract of moribund fish were sampled and fixed in 10% neutral buffered formalin (NBF) and processed for histopathology. Tissues were processed, embedded in paraffin wax, where 5µm thick sections were cut and stained using haematoxylin and eosin (Lillie 1965). The stained tissue sections were viewed for histopathology analysis while Gram staining of tissues (Brown & Hopps 1973) was used for detecting the presence of the bacteria in the affected organs. At the end of the challenge period, 50% of all surviving fish per treatment group were removed and examined for gross clinical signs of disease and sampled for bacterial recovery as already described. All experiments were conducted with the approval of the University of Stirling Ethics Committee and performed under Home Office Licence 60/3949.

3.3.6. Statistical analysis

Parametric assumptions were checked using Levene´s test for homogeneity of variances and Shapiro-Wilk´s test for normality. The samples with homogenous variances were analyzed using the Duncan test, while Dunnett´s T3 test was used for the samples with unequal variance. As data were normal-distributed and homoscedastic, the cumulative percentage mortalities of 3 replicates between treatment groups were compared by using one-way ANOVA, followed by the Duncan test. All the tests were performed using the SPSS program release 17.5. Differences were tested at a level of significance of 5%.

3.4. Results

3.4.1. Cumulative mortality percentage of challenge experiment.

The cumulative percentage daily mortalities for the fish exposed by immersion per exposure time were provided in Figure 3.1. Mortalities were observed in all fish groups receiving the bacteria (Figure 3.1). The highest total mortalities (100%) were found in groups that had been exposed to the bacteria for 5 min or longer (Figure 3.1). In addition, fish exposed to bacteria in the longer immersion time groups died more rapidly when compared with those exposed for 1-2 min (Figure 3.1). The mortality was considered too high using these exposure times and so a second immersion study was performed with shorter exposure times (Figure 3.2).
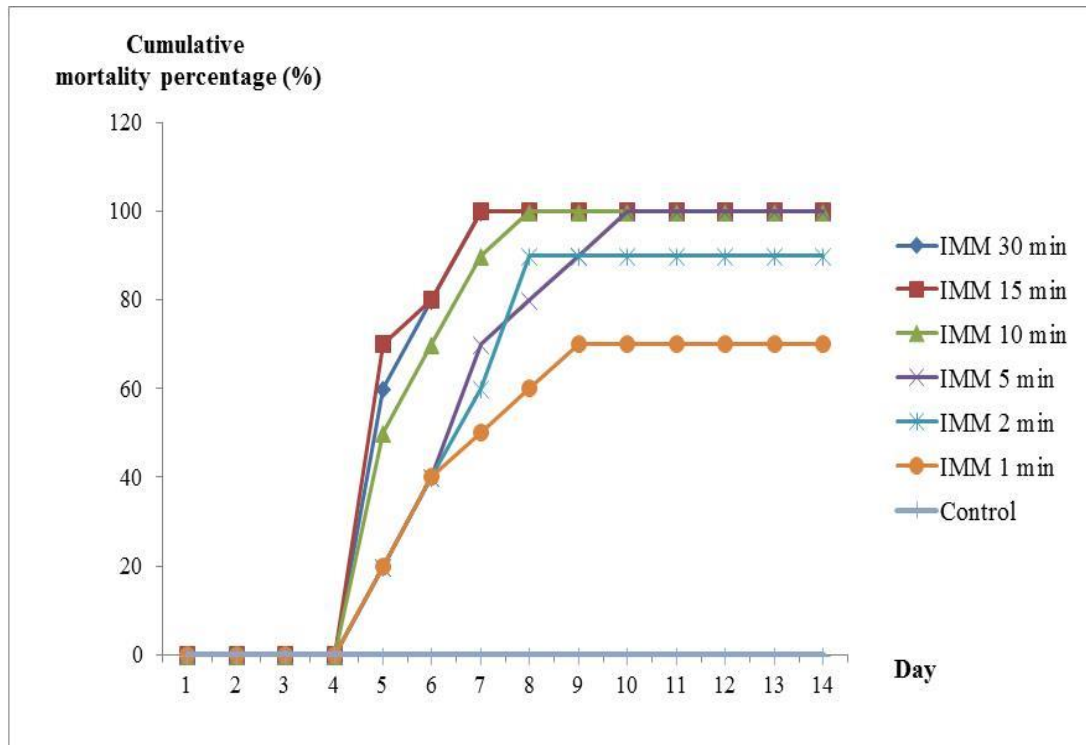
Figure 3.1. Cumulative percentage mortalities in the pre-challenge immersion experiment. Fish was exposed at 10^7 cfu ml^{-1} of *E. ictaluri* for 1 min to 30 min (IMM = immersion)

The cumulative percentage daily mortalities in the refined immersion challenge study are presented in Figure 3.2. There were no mortalities in the control group throughout the study period (Figure 3.2). The first mortality occurred at day 3 within the group exposed for 2 min (Figure 3.2) and the second mortality was observed in the treatment group exposed for 1 min at day 4 post bacterial challenge. The duration of this immersion challenge had an effect on time to death between treatments from day 3 to day 4 post challenges (Figure 3.2). However, from day 5 post-bacterial exposure the mortalities occurred in all treatment groups except the control (Figure 3.2). The highest percentage cumulative mortality was found in the treatment group exposed to

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the bacteria for the longest duration (2 min, Figure 3.2). The mortality curves for each of the treatment groups were similar (Figure 3.2).
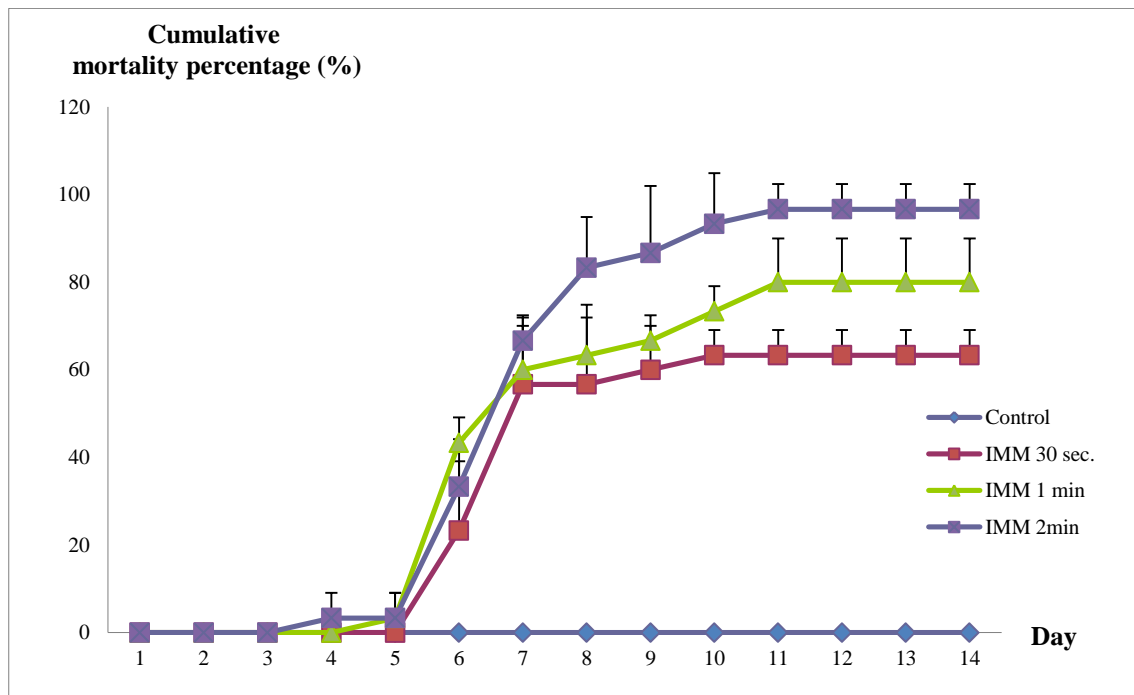
Figure 3.2. Cumulative percentage mortalities in the immersion challenged groups with *E. ictaluri* for 30 second (IMM 30 sec), 1 min (IMM 1 min), and 2 min (IMM 2 min) compared with the control group.

Although the cumulative mortality was higher in the group exposed to bacteria for 2 min than in the other challenge treatment, no statistically significant difference was noted between treatments from day 4 to day 7 (p = 0.310 compared with treatment group exposed for 30 sec and p= 0.488 compared with treatment group exposed for 1 min). From day 8 onwards, mean percentage mortality in the 2 min immersion group was only significantly higher than the 30 seconds immersion group (p = 0.045) and was significantly higher than the 1 min immersion group (p = 0.024) on day 10. Meanwhile, the mean of the cumulative mortality percentage in the 1 min group was higher than the 30 second immersion group, however, the significantly higher value was only

observed on day 11 post challenge (p = 0.34). High mortality was similar between replicate tanks per treatment group from day 4 to day 8. The end-point mortality (63%) was found in groups that had been exposed to the bacteria for 30 sec (Figure 3.2).

3.4.2. Cumulative mortality percentage of cohabitation experiment

Cohabitation challenge with infected fish by i.p. had a higher cumulative mortality percentage than cohabitant by immersion infected fish (p = 0.013) (Table 3.4).

Table 3.4. Mortality among groups of challenged striped catfish with various controls or *E. ictaluri* infection followed by a cohabitation challenge. The groups are identified where the challenge route used to infect seed fish with *E. ictaluri* was by intraperitoneal (i.p.) injection or immersion (IMM). The first mortality was recorded as day post-challenge (DPC)

Treatment group	The seed fish		Number of un-infected fish	First mortality (DPC)	Final mortality (%)
	Concentration of *E. ictaluri*	Route/ exposure time			
1	1.08×10^7 cfu ml^{-1}	IMM for 15 min	9	7	22
2	1.08×10^7 cfu ml^{-1}	IMM for 15 min	9	7	22
3	1.08×10^6 cfu fish^{-1}	i.p.	9	5	33
4	1.08×10^6 cfu fish^{-1}	i.p.	9	5	44
Control 1	0	IMM for 15 min (0.85% sterilized saline water)	9		0
Control 2	0	i.p. with 0.85% sterilized saline water	9		0

Control 1: control group of cohabitation with infected seed fish by immersion; Control 2: control groups of cohabitation with infected seed fish by i.p.

Immersion cohabitation groups showed a slower pattern of mortality compared with i.p. infected cohabitant fish. The first mortality was detected at day 5 in the treatment group of i.p. challenged cohabitants. In the treatment group where the seed fish were

exposed to *E. ictaluri* for 15 min and kept with healthy fish, the first mortality occurred on day 7. No mortalities or morbidity were observed in the seed saline/control fish or any other fish in the same treatment group (Table 3.4).

3.4.3. Clinical signs and gross pathology

Within 3 to 4 days post exposure clinical signs commonly associated with *E. ictaluri* infection were observed in the fish in both immersion challenges and at day 7 in the cohabitation experiments (Figure 3.3 & 3.4). Affected fish in both immersion and cohabitation experiments showed behavioural changes including erratic swimming in a spiral motion and stopped feeding prior to mortality. Generally, the affected fish had few clinical signs of disease externally with the exception of some fish showing petechial haemorrhages in the fins and eyes. The anus of some fish (12/96 fish) was swollen, protruding and deep red in colour. Skin lesions with faded pigment nodules were observed in all challenging routes; however the presence of focal skin lesions (Figure 3.3 A & B) in the fish was only recognised after day 7 when the mortality reached the highest level.

Internally, the affected fish presented grossly with white lesions (1-2 mm diameter) observed distributed throughout the spleen and the kidney (Figure 3.4). Later, white lesions also occurred on the liver of infected fish. The body cavity was swollen and ascites was present. Spleen and kidney were enlarged. Pure cultures of bacteria identified as *E. ictaluri* were only recovered from moribund and fresh dead fish.

No mortalities/morbidity, clinical signs of disease or bacteria were observed or recovered from the control group or any of the survivors.

Figure 3.3. (A). Normal skin of un-infected striped catfish with *E. ictaluri.* (B) The presence of small focal circumscribed non-haemorrhagic lesions present on the skin (circle) was observed in the skin of fish infected with *E. ictaluri*
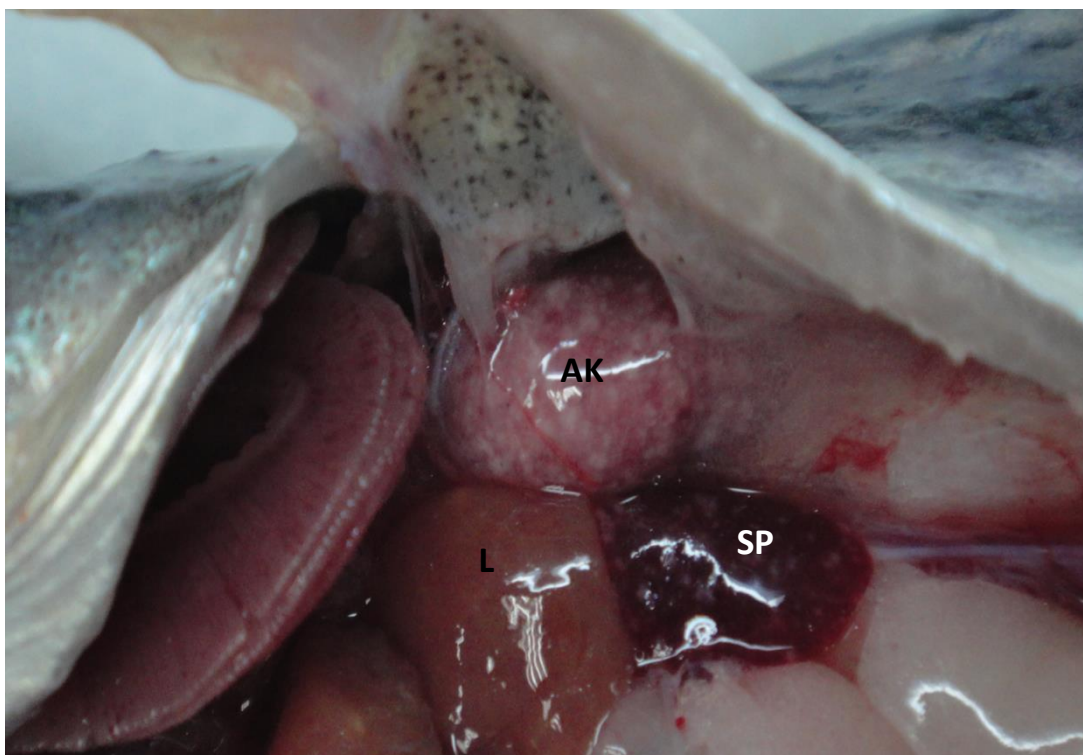
Figure 3.4. White lesions were presented in the anterior kidney (AK) and spleen (SP). Liver (L) was enlarged.

3.4.4. Phenotypic and genetic identification

The isolated strains from 96 moribund and fresh dead fish recovered during the challenge studies showed almost identical phenotypic characteristics with the original challenge strain. By contrast, mixed colonies were observed with fish that were removed sometime after death but only one sub culture was required for pure bacterial recovery from the dominant colony morphology. They all gave Gram negative, non-motile short or varied length rods, fermentative on O/F and oxidase negative with an API 20E profile of 4004000. This bacterium gave β-haemolysis on sheep blood agar. The typical triple sugar iron agar reaction showed that no H_2S, acid, gas or slight gas were produced. This bacterium was negative for citrate, urea, VP,

indole, gelatine, oxidase, arginine dihydrolase, ornithine decarboxylase and β-galactosidase.

The biochemical characteristics of the isolates recovered were identified with a battery of biochemical reactions and tests that are summarised in table 3.4. Results from Waltman et al. (1986) for USA *E. ictaluri* recovered from ESC infected channel catfish are included for comparative purposes. Challenge and recovered strains gave a negative result for ornithine decarboxylase reaction. These isolates were positive for lysine decarboxylase and fermentative on O/F test. Of the nine carbon-sugars tested, only fermentation was observed using glucose as the substrate (Table 3.5).

In all, the phenotype of the isolated strains from 96 moribund and dead fish all presenting with clinical signs of BNP was consistent with the other members of the genus *Edwardsiella* and was identified as *E. ictaluri*. Rate of re-isolation in moribund and dead fish of the bacterial group was 100%. Additionally, no pathological findings were observed and no bacteria were recovered from the surviving fish in any of the treatment or the control groups of fish at the end of the study period.
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All of the *E. ictaluri* strains recovered from the experimentally exposed fish expressing clinical signs of BNP were confirmed positive by PCR as they provided a single molecular weight band recognised at 470 bp (Figure 3.5).

Table 3. 5. Biochemical characteristics of strain used in the challenge and recovered strains during challenge in comparison with the strain used in the study of Waltman et al. (1986).

Substrate test	Experimental E. ictaluri	Recovered isolate	E. ictaluri (Waltman et al., 1986)
Gram stain	-	-	-
Motility	-	-	-
Cytochrome Oxidase	-	-	-
Hemolysis	β - hemolytic	β - hemolytic	β - hemolytic
β-galactosidase (ONPG)	-	-	-
arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	-	-	+
Citrate	-	-	-
H$_2$S	-	-	-
Ure	-	-	-
Indole	-	-	-
Tryptophane Deaminase	-	-	-
Voges-Proskauer	-	-	-
Gelatine	-	-	-
Glucose	+	+	+
Mannose	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Saccharose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Arabinose	-	-	-

470 bp
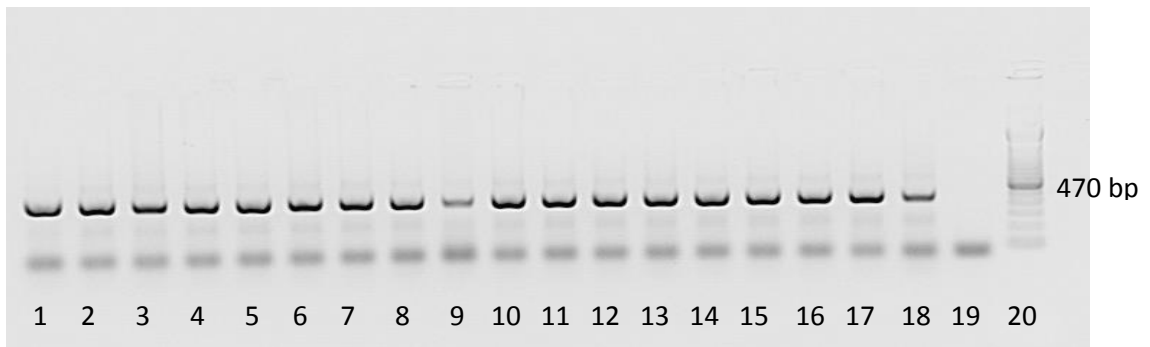
Figure 3.5. *Edwardsiella ictaluri* PCR products resolved by agarose gel electrophoresis showing lane 1 to 16: templates prepared from recovered isolates during challenge test, lane 17: template prepared from isolate used for challenge, lane 18: *E. ictaluri* NCIMB 12733 (positive control); Lane 19: *E. tarda* (negative control); Lane 20: 1 kb ladder

3.4.5. Histopathology

Large areas of cellular necrosis and haemorrhage were present in the spleen and kidney from the moribund fish sampled. Multiple extensive areas of necrosis were observed particularly in the anterior kidney (Figure 3.6).

Haemorrhages were observed in kidney, spleen and hepatopancreas. Small colonies of Gram-negative rods were observed in the kidney and spleen of moribund infected fish (Figure 3.7).

The spleen also showed extensive confluent areas of necrosis within the parenchyma. Gill showed moderate lamellar fusion and displayed moderate, multifocal hyperplasia and occasional areas of necrosis. Several medium sized blood vessels in the serosa of the intestinal tract were obliterated by fibrin thrombi and their walls were infiltrated by small numbers of inflammatory cells (vasculitis). A large aggregate of fibrin was seen in the peritoneal cavity. Focal areas of circular non-haemorrhagic lesions were seen in skin.
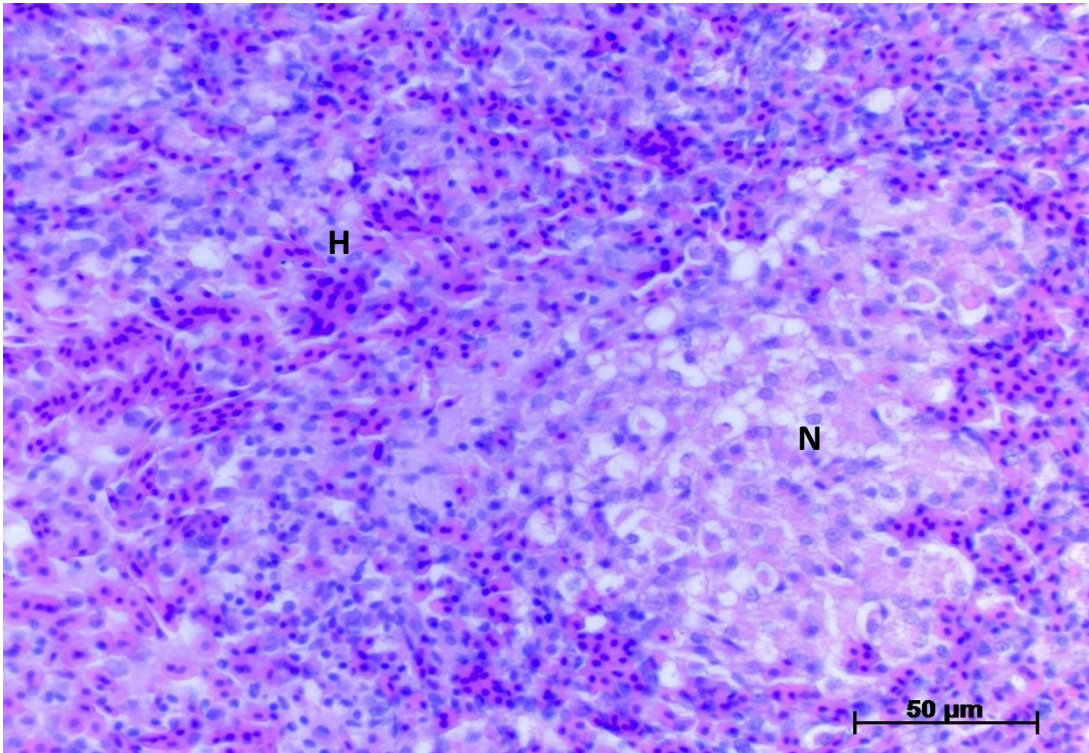
Figure 3.6. Extensive area of necrosis (N) and haemorrhage (H) in anterior kidney (H&E)
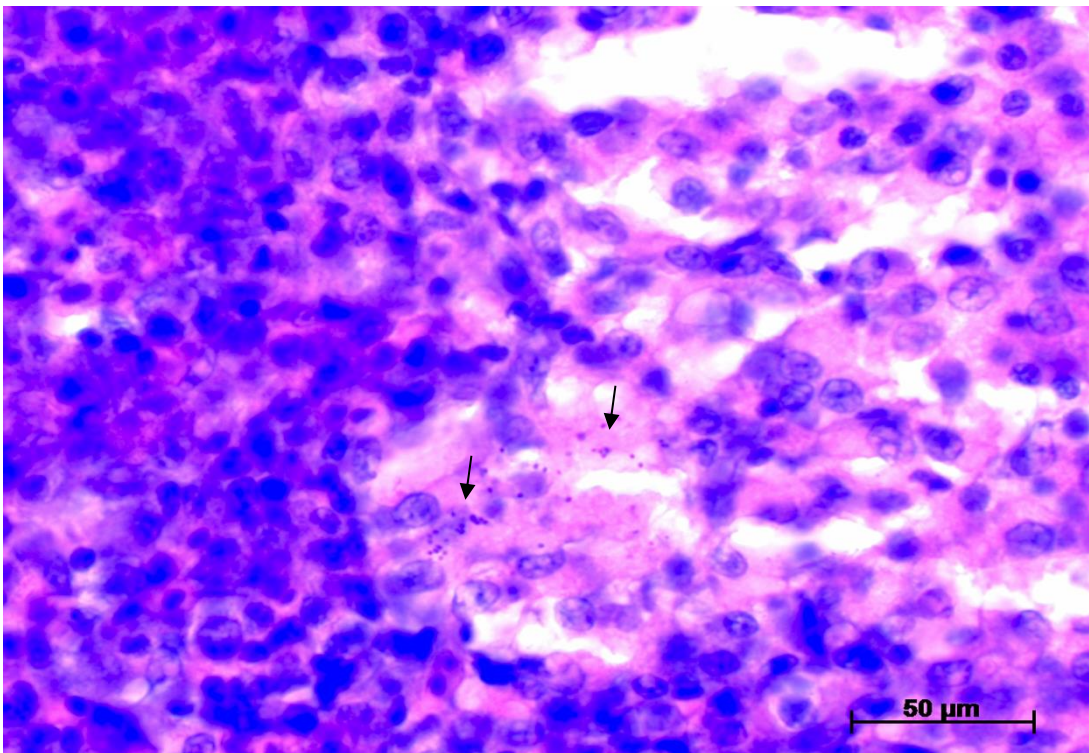
Figure 3.7. Extensive areas of necrosis, haemorrhage and small colonies of Gram-negative rods (arrows) in the spleen (H&E; x100)

3.5. Discussion

The results of this study produced a successful immersion challenge model for *E. ictaluri* in *P. hypophthalmus*. The bacterium recovered and identified from the affected fish during the challenge study was identified as *E. ictaluri,* through primary, biochemical and PCR methods applied in this study. This challenge experiment demonstrated that the infection of *E. ictaluri* was confirmed according to Koch's postulate as shown previously (Crumlish et al. 2010). Comparison of the data provided in this study showed that the fish exposed to the bacterium showed similar behavioural and clinical signs to those described for both natural and experimental BNP infections (Ferguson et al. 2001; Crumlish et al. 2002; Ho et al. 2008; Crumlish et al. 2010).

Externally, petechial hemorrhagic lesions of the skin are characteristic during ESC outbreaks in infected channel catfish, which are more obvious (Hawke et al. 1981) in the early or acute stage of ESC in channel catfish compared with the open lesion or "hole in the head" which develops in the later or chronic stage of the disease (Plumb & Vinitnantharat 1993). Petechial haemorrhages have been observed in the fin, eye and the anus of some experimentally infected *P. hypophthalmus*. Skin lesions associated with BNP infections in *P. hypophthalmus* have not been described previously in the literature, but in this study they developed in the affected fish towards the end of the challenge period and were similar to those observed in previous studies performed in this challenge facility (Crumlish pers. com.). On the fish farms in Vietnam such skin lesions have only been reported verbally and samples have

not been observed. They are certainly not commonly associated with this disease in this fish species. It may be that continual exposure to virulent strains of *E. ictaluri* in the water may provide these additional external clinical signs, however further study is needed on this issue.

These additional skin lesions in the striped catfish are similar to the more chronic form of ESC that is described in other fish species (Hawke et al. 1981; Pasnik et al. 2007; Ye et al. 2009). No external lesions were obtained in any of the fish that died in the first period of infection, which was similar to the first stage of *E. ictaluri* infection in striped catfish. The lack of skin lesions from the onset of the infection conducted in this study may be due to insufficient time for infected fish to develop the clinical symptoms before death. This pattern correlated well with the onset of BNP infections reported on the farms where there are high mortality and the fish die rapidly so they do not have time to develop skin lesions, however further study is needed.

Bacterial recovery from the spleen and kidney samples of all infected striped catfish (both moribund and dead fish) produced bacterial colonies identified as *E. ictaluri*. The histopathology taken from moribund fish exposed to the bacteria and with gross clinical signs of BNP was also similar to that observed in previous natural and experimental infections in BNP studies (Ferguson et al. 2001; Crumlish et al. 2002; Crumlish et al. 2010). The aim of the present study was to produce an immersion infection model, which was considered to be more natural compared with injection routes (Bell et al. 1984; McCarthy et al. 1984; Madsen & Dalsgaard 1999). The

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present data showed that both immersion and cohabitation exposure were successful models which can be used for studying the pathogenicity of *E. ictaluri* infections in striped catfish. While the i.p. challenge route is useful, it bypasses the non-specific cellular and specific immune mechanisms located in the skin. Immersion and co-habitation methods are considered to be optimal for studying fish infections as they more closely mimic the natural exposure to pathogens by non-bypassing the natural skin and mucosal barriers (Madsen & Dalsgaard 1999). It is likely that the conditions during the immersion challenge were reflecting those found in aquaculture environments.

In the immersion challenge performed in this study, mortality rates proved to be a dependent on the challenge dose received (Murray et al. 1992). In the present study, mortalities were obtained in all treatment groups except the controls. During the passage experiments with *E. ictaluri* isolates recovered from BNP outbreaks in Vietnamese striped catfish, all fish which received bacteria by i.p. were dead in one or two days and bacteria were recovered from the kidney and spleen regardless of the strain used, suggesting that all Vietnamese *E. ictaluri* isolates may have the ability to colonise internal organs of striped catfish. The pre-challenges highlight the strong virulence of the strain used in this study. Fish which received this isolate expressed the typical clinical signs consistent with those observed in other studies (Crumlish et al. 2002; Crumlish et al. 2010), mortalities were 100% even at the short immersion time (5 min) and pure bacterial colonies were recovered from the kidney of all moribund

fish and fresh dead fish. These observations showed that this isolate is virulent to striped catfish.

Other experimental challenge studies performed in striped catfish using the same immersion route presented mortalities as high as in the present study by using prolonged immersion time for 30 minutes to 1 hour. However, the fish are also subjected to the additional stress of handling by prolong exposing fish in the pathogen (Alcorn et al. 2005). Immersion in 1.2 x 10^6 cfu ml^{-1} of *E. ictaluri* for 1 hour caused 100% mortality of yellow catfish (Ye et al. 2009). The LD$_{60}$ of *E. ictaluri* for striped catfish was 1x 10^6 cfu ml^{-1} for 1 hour immersion and 3.5 x 10^6 cfu ml^{-1} in ip-injected fish (Murray et al. 1992; Thinh et al. 2009). Another study reported that an immersion challenge dose of 1 x 10^8 cfu ml^{-1} for 1 hour or 1 x 10^6 cfu ml^{-1} in i.p.-injected fish gave more than 80% control mortality (Crumlish et al. 2010). However, only the study of Crumlish et al. (2010) regarded the isolate, which was used in challenge experiments as non-haemolytic. In comparison, striped catfish challenged with lower concentration of β- haemolysin *E. ictaluri* gave an end-point mortality of 60% and 80% where the fish were exposed to the 1 x 10^7cfu ml^{-1} of *E. ictaluri* for 30 seconds or 1 minute respectively. The end-point mortality increased to close to 100% as the exposure time increased to 2 min over the expected end-point mortality. These results showed that the virulence of this haemolytic strain is extremely high compared with other isolates used in the previous studies of Thinh et al. (2009) and Crumlish et al. (2010). However, haemolysin has been suggested not to be essential for virulence of *E. ictaluri* in catfish (Williams et al. 2003; Williams & Lawrence 2005).

The results of this study showed that *E. ictaluri* can establish and cause disease in striped catfish even after a short exposure time and this bacterium was able to cause disease when administrated experimentally. Short-term exposure of fish to bacteria avoids unnecessary stress and as this is a more natural exposure route can help elucidate the mechanism of bacterial infection and disease establishment in fish.

The intestine, olfactory sinus and gills have all been considered as entry sites for *E. ictaluri* into channel catfish (Newton et al. 1989; Baldwin & Newton 1993; Morrison & Plumb 1994; Nusbaum & Morrison 1996). Recently, Menanteau-Ledouble et al. (2011) reported that *E. ictaluri* entered channel catfish through the skin instead of penetrating the fish through intestine, nares, or gills. They also mentioned that *E. ictaluri* was able to invade catfish through abraded and intact skin. Trust (1986) reported that fish pathogens can penetrate fish via skin, gill or occasionally the oral route. Although the results from this study did not investigate the entry site of the bacteria into the striped catfish, the short exposure time of 30 seconds was sufficient to establish an infection as shown from the clinical signs, mortalities, bacterial recovery and histology performed in this study. It might be that the first encountered route of entry such as skin is more a matter of opportunity rather than actual tissue specificity.

The experimental challenge has to be terminated within the incubation period when the mortality of infected fish was caused only by the original inoculation and not spread between fish in the system (Nordmo & Ramstad 1997). In the case of Atlantic

salmon (*Salmo salar* L.) infected with *Aeromonas salmonicida* by i.p. or immersion, the incubation period was five to six days (Nordmo & Ramstad 1997). In this period some fish died from the original inoculation and others died from a water-borne infection set up in the tanks by the inoculated fish. After that the challenge can no longer be considered as pure i.p. or immersion because a second rise was observed five to six day later after the daily mortality fell to a lower level. Nordmo & Ramstad (1997) suggested that the i.p. or immersion challenge of *A. salmonicida* in Atlantic salmon would have to terminated in 10-12 days at the temperature of 10-12°C to avoid super-infection.

However, according to results of the series of pre- challenge tests carried out in this study, the second rise of mortality has not occurred after the initial mortality lasting for five or six days in the experimental challenge of striped catfish with *E. ictaluri* by i.p. or immersion (un published data). The mortality rates were expected to increase in such a manner that the experiments could be terminated with mortality of challenged fish exceeding 60%. Unfortunately, 60% mortality was not attained in any cohabitation challenge groups, the expected mortality was only observed in immersion challenge experiments. However, the mortality of striped catfish exposed to *E. ictaluri* in both immersion challenge and cohabitation challenge experiments stopped at day 12, and mortality was used as the end point at day 14 which follows the suggestion of Amend (1981) which defined the end point for the challenge experiment as two days beyond the day that the last fish specifically died from the infection.

Cohabitation challenge permits the determination of crossover infections within a group of infected and un-infected fish (Murray et al. 1992). However, the cohabitation challenge takes a significantly longer time between the introduction of the infected seed fish and the onset of mortality among the challenged fish than by immersion (Alcorn et al. 2005). Physical contact is considered as a risk factor for transmission of any pathogen in the water body (Cvitanich et al. 1991). Whilst the results of the co-habitation method developed in this study clearly showed mortalities with clinical signs of disease and recovery of the infectious agent, it was difficult to determine the challenge dose received by the crossover fish. The first requirement of virulence in horizontal transmission of pathogens is their ability to survive in aquatic environments, to penetrate a susceptible host (Trust 1986). According to the study of Plumb & Quinlan (1986), *E. ictaluri* was found to survive in the pond bottom mud for 40 days at 18°C and for 95 days at 25°C. However, in water *E. ictaluri* survived for less than 10 days at 25°C and for less than 15 days at 5°C. Nevertheless, our data showed that it is possible to achieve infection through cohabitation where the seed fish were challenged by i.p injection or through immersion. These results presumed that contact between infected fish and non- infected ones might play an important role in the horizontal transmission of *E. ictaluri* in striped catfish ponds where the infection spreads rapidly to healthy fish in the same pond and contiguous ponds once the BNP occurs.

Some studies also point out the importance of close contact between infected fish and un-infected fish in transmission of *E. ictaluri* in channel catfish (Shotts et al. 1986; Klesius 1994; Gaunt et al. 2006) or in some bacteria such as *Renibacterium*

salmoninarum in chinook salmon (Murray et al. 1992) or *Piscirickettsia salmonis* in fresh water-raised Atlantic salmon (Almendras et al. 1997). In the present study, the mortality of striped catfish in the cohabitant by i.p. or immersion (39% and 22% respectively) confirmed the importance of physical contact as a means of spread in horizontal transmission of *E. ictaluri* among striped catfish thus early removal of infected fish might be important in reducing the infection of *E. ictaluri* in striped catfish at the farm level potentially important information for control. A large number of fish in the pond leads to an increase in the infection of *E. ictaluri* in striped catfish. Furthermore, the high density of striped catfish applied in grow out farming (Phan et al. 2009; 2011) can cause an increase in severity of infection with this bacterium.

The present study fulfilled the aims and produced two non-injection challenge models: immersion and cohabitation. The immersion and cohabitation challenge method obviously has the disadvantage of taking considerable time to be achieved because of the slow-growth and slow colonization of the fish by fewer bacteria (Bell et al. 1984; McCarthy et al. 1984; Murray et al. 1992), and is more difficult to control and standardize than the injection method (Madsen & Dalsgaard 1999). Cohabitation challenge is considered the most natural infection of an uncompromised host and a virulent pathogen because it eliminates the disadvantage of immersion challenge where the fish are typically exposed to unnaturally high concentrations of bacteria (Alcorn et al. 2005). Both of these methods would be suitable for investigating pathogenesis of *E. ictaluri* infections in *P. hypophthalmus*. However, the results of this study showed that although the mortality occurred at day 5 or day 7 after introduction

of the infected seed fish to the test fish, the end-point mortality did not increase as expected in any of treatment groups (60%). By contrast, an adequate level of challenge was achieved in the immersion challenge, which provided a minimum of 60% mortality of the infected fish. The immersion is thought to be a more suitable model for use in establishing BNP clinical infection during experimental studies. The present study also confirms the horizontal transmission of *E. ictaluri* in striped catfish.

3.6. References

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CHAPTER 4. Environmental conditions affecting infection of *Edwardsiella ictaluri* in striped catfish *Pangasianodon hypophthalmus* (Sauvage)

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In this chapter the link between environmental factors and the infectivity of *E. ictaluri* was investigated both in *in vitro* tests and *in vivo* tests by the candidate under the supervision of all co-authors. The candidate designed the study and conducted the experiments at the Wet laboratory, Ho Chi Minh International University, Vietnam. Mr Dang Ngoc Phuong, Dr Hoang Tung, Dr Nguyen Minh Thanh and Ms Vo Thi Minh Thu (Ho Chi Minh International University) provided the assistance to implement the *in vivo* tests.

The text of this chapter is presented as a publication-ready manuscript. Dr Mags Crumlish and Prof Randolph Richards provided supervisory and editorial support throughout the whole study.

CHAPTER 4. ENVIRONMENTAL CONDITIONS AFFECTING INFECTION OF *Edwardsiella ictaluri* IN STRIPED CATFISH *Pangasianodon hypophthalmus* (Sauvage)

4.1. Abstract

In this study, the growth of the bacterium identified as *E. ictaluri* was investigated *in vitro* when cultured under different pH and salt concentrations. The results showed that a pH value between 5.5 to 6.5 and salt concentration between 0-0.5% was optimal for the growth of *E. ictaluri*. The effect of varied pH and salt concentrations on the susceptibility of striped catfish to *E. ictaluri* infection was also studied *in vivo* following a bacterial immersion challenge. The catfish were immersed in 1.06 - 1.1 x 10^7 cfu ml^{-1} *E. ictaluri* for 30 seconds and kept in varied pH and salt concentrations. The cumulative mortality of striped catfish in water at pH 5 and pH 6 was significantly higher than that of fish maintained in more alkaline water ($p<0.05$). By contrast, the cumulative mortality of the striped catfish maintained in 0.5% salt concentration was significantly lower than those kept in 0%, 1 % and 1.5 % salt concentration ($p<0.05$). The histopathology also showed hypertrophy of the skin and hyperplasia of the gill epithelium of striped catfish under conditions of low pH and high salinity. This study identified the potential value of pH 8.5 and salinity of 0.5 % NaCl as a means of decreasing the susceptibility of striped catfish to *E. ictaluri*.

Keywords: *Edwardsiella ictaluri, Pangasianodon hypophthalmus,* environmental conditions, pH, salinity.

4.2. Introduction

The role of environmental factors in influencing clinical outbreaks of *E. ictaluri* in fish has been studied in the USA channel catfish mostly through experimental infections. Changes in water temperature outwith the range (25-30°C) was the factor that has often been reported as a precursor to establishment of *E. ictaluri* infection in channel catfish (Walters & Plumb 1980; Francis-Floyd et al. 1987; Baxa-Antonio et al. 1992; Plumb & Shoemaker 1995). Increased salinity affects the host response by increasing the activity of lytic enzymes, respiratory burst of macrophages and the circulating levels of IgM (Uribe et al. 2011), but also generates a more favourable osmotic environment leading to an increased burden of pathogens (Bowden 2008). In addition, a high concentration of NaCl (3%) was shown to induce haemagglutination activity leading to enhancement of the virulence of pathogens (Park et al. 2012). However, it is reported that the mortality during natural infections with *E. ictaluri* decreased in low salinity conditions (Plumb & Shoemaker 1995).

Bacterial diseases have been reported as the major infections affecting Vietnamese striped catfish farming (De Silva & Phuong 2011), where outbreaks can account for up to 50% of total losses in these farms compared with other infectious diseases (Phuong et al. 2007). Bacillary necrosis of Pangasius (BNP) is a bacterial infection caused by *Edwardsiella ictaluri* (Crumlish et al. 2002) and is considered as a most serious disease occurring in striped catfish farms (Crumlish & Dung 2006; Phan et al. 2009; De Silva & Phuong 2011; Phan et al. 2011; Luu 2013). Whilst BNP infections can occur in these production systems throughout the cycle, this disease has been reported to occur seasonally as more severe infections (as shown by total fish losses) appeared in water

temperatures between 25-30°C, which correlated with the onset of the wet season, and increased rainfall in Vietnam (Dung et al. 2004; Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). This temperature range was found to be optimal for the growth of *E. ictaluri in vitro* (Plumb & Vinitnantharat 1989) and temperatue correlated well with the bacterial infection in fish (Baxa-Antonio et al. 1992).

Optimal environmental conditions in the farms of striped catfish are required but may be degraded resulting in pH changes, fluctuating water temperatures and even pollution: all of which have been associated with the occurrence of this disease in striped catfish (Phuong et al. 2007). However, the effect of environmental conditions on the establishment of *E. ictaluri* infections in farmed striped catfish has not been fully investigated. Thus, the aims of this study were to investigate the tolerance range of pH and salinity on viability of *E. ictaluri in vitro* and then to explore these ranges on the disease susceptibility through *in vivo* experimental infection in *P. hypophthalmus*.

4.3. Materials and methods

4.3.1. Biophysical tests on pH and NaCl tolerance of *E. ictaluri*

A total of 36 *E. ictaluri* isolates recovered from 36 clinical disease outbreaks in four provinces in Vietnam (Vinh Long, Can Tho, An Giang and Dong Thap province) from 2002 to 2011 were investigated for growth characteristics when grown in varied concentrations of sodium chloride and pH values. These isolates were selected and representative of the six groups identified from the Pulsed Field Gel Electrophoresis (PFGE) study and for 4 provinces (chapter 2) (Table 4.1). The *E. ictaluri* type strain 12733 purchased from the National Collection of Industrial and Marine Bacteria

(NCIMB) was used as an internal control. All isolates from 2002-2009 were collected
and provided by Dr. Crumlish (IOA, Stirling).

Table 4.1. List of *E. ictaluri* isolates according to the geographical region, year of isolation and
fingerprinting (PFGE) groups.

PFGE groups	Isolate ID	Province	Year	Number strains /group
A	020	Dong Thap	2004	10
	021	Can Tho	2004	
	046	Dong Thap	2004	
	044	Dong Thap	2006	
	055	Dong Thap	2009	
	063	An Giang	2010	
	066	An Giang	2010	
	070	An Giang	2011	
	071	An Giang	2011	
	079	Vinh Long	2011	
B	001	Dong Thap	2002	10
	024	An Giang	2002	
	012	Dong Thap	2003	
	013	Dong Thap	2003	
	029	Dong Thap	2005	
	038	An Giang	2005	
	023	Dong Thap	2006	
	042	An Giang	2006	
	034	An Giang	2007	
	045	Dong Thap	2007	
C	005	An Giang	2002	6
	008	Can Tho	2002	
	018	An Giang	2004	
	049	An Giang	2008	
	073	Vinh Long	2011	
	074	Vinh Long	2011	
D	022	Dong Thap	2007	6
	059	Dong Thap	2008	
	060	Dong Thap	2008	
	054	Dong Thap	2009	
	062	Can Tho	2010	
	076	Dong Thap	2011	
E	026	Dong Thap	2005	2
	037	Vinh Long	2005	
F	036	Vinh Long	2005	2
	040	Vinh Long	2006	
Total				**36**

4.3.1.1. Bacterial preparation

From a pure bacterial growth plate on Tryptone Soya Agar (TSA, Oxoid, England), a single colony was removed and placed directly into 5 ml of sterile Tryptone Soya Broth (TSB, Oxoid, England) and incubated overnight at $28^{o}C$ in the shaking incubator (Kuhner shaker, ISF-1-W, Switzerland; 140 rpm). After 24h the bacterial suspension was centrifuged at 3,500 rpm (Sanyo NSE Mistral 2000R, Japan) and the cell pellet re-suspended to give an OD 600nm value of 1 using 0.85% sterile saline solution. The Miles & Misra method provided viable colony counts (Miles et al. 1938) and then 10-fold serial dilutions were performed to give approximately 1×10^7 cfu ml^{-1}. This was the bacterial concentration used for all of the biophysical assays performed in this study.

4.3.1.2. Salt tolerance assay

One hundred microliters of pure *E. ictaluri* suspension at (10^7cfu ml^{-1}) was aseptically inoculated into 30 ml of sterile TSB with 6 salt concentrations (0, 0.5, 1.0, 1.5, 2.5 and 4.0 % NaCl) and grown in a shaking incubator (Kuhner shaker, ISF-1-W, Switzerland) at $28^{o}C$, 140 rpm for 24 hours. Salt tolerance of the isolates was defined for growth when inoculated in 0.0, 0.5, 1.5, 2.5 and 4.0 % NaCl in TSB broth while 0.5% NaCl was used both as test-broth and control-broth. The un-inoculated TSB broth was used as the negative control group (Plumb & Vinitnantharat 1989; Benson 2002). Each salt tolerance assay was performed with 3 replicates. After 24 hours, the optical density (OD$_{600nm}$) was measured and viable colony counts were performed.

4.3.1.3. pH tolerance assay

Farm data on the pH ranges in the striped catfish ponds both outwith and during disease outbreaks were used as a guide for the assay performed (unpublished data). The pH range of 4.5, 5.5, 6.5, 8.5 and 9.5 was used. The samples were incubated as described in section 4.2.1.2 while the pH of TSB (7.3) and un-inoculated tubes of TSB were used as an internal and a negative control respectively. The pH values of TSB were adjusted using 1 N HCl or KOH (Oxoid, UK) and measured by pH metter prior to inoculation. Each pH tolerance assay was conducted with 3 replicates. The densities of all strains under different pH values were defined after incubating for 24 hours by spectrophotometry (OD_{600nm}) and viable colony counts performed.

4.3.2. Experimental infection of *E. ictaluri* in striped catfish under different pH and NaCl concentration.

4.3.2.1. Fish

This work was performed using stock fish within the Applied Hydrobiology Laboratory of the International University, Ho Chi Minh National University, Vietnam. These fish were originally from The National Breeding Centre for Southern Freshwater Aquaculture at An Thai Trung Commune, Cai Be district, Tien Giang province, Vietnam. The fish had been quarantined in the wet lab of the International University for 14 days prior to use. These stocks were maintained in 4000L fibreglass tanks at $28^{o}C \pm 2^{o}C$, and fed commercial catfish diet (Catfish 2 T502, Uni-President Co., Vietnam). Fish used in this study were 15-20g in weight.

4.3.2.2. Bacterial challenge

A bacterial strain of *E. ictaluri* recovered from a natural outbreak of BNP in Vietnamese *P. hypophthalmus* was used for all challenge studies. This isolate was obtained from Dr. Crumlish, University of Stirling and stored in commercially prepared cryoprotect beads (Technical Service Consultant Ltd, UK) at -70°C until use. This isolate was identified as *E. ictaluri* and passed through fish twice as previously described in chapter 3.

All challenge inocula were prepared as described in 4.2.1.1 where the resulting challenge dose of approximately 1×10^{7} cfu ml^{-1} were used. Fish were immersed for 30 seconds in 10L tanks containing bacteria at 1.06×10^{7} cfu ml^{-1} and then removed and placed in the flow-through experimental tanks and observed for 14 days. Immersion concentrations were chosen based on a series of pre-challenge studies performed previously at the Aquaculture Research Facility (ARF), University of Stirling and also at the Applied Hydrobiology Laboratory of the International University, Ho Chi Minh National University, Vietnam (data not shown).

4.3.2.3. Experimental design of the effect of pH on the *E. ictaluri* infection in *P. hypophthalmus.*

Bacterial preparation

The bacterial preparation was as described above except that the bacteria had been cultured in TSB medium at pH 5.5, 6.5, 7.5 or 8.5.

Experimental design

Four treatment groups of 10 fish in triplicates (n= 120) were maintained in water at either pH at 5.5, 6.5, 7.5 or 8.5 (Table 4.2) for 2 weeks before challenge with the *E. ictaluri* grown at pH 5.5, 6.5, 7.5 or 8.5. The pH values of water were adjusted using 1 N HCl or KOH (Oxoid, UK) before supplying to the challenge tanks. The aquaria water was maintained at pH 7.5 and served as control receiving the same treatment but not bacteria.

Table 4.2. Experimental design of effects of pH on the *E. ictaluri* infection in *P. hypophthalmus*

Treatment group	pH of water	No.fish/tank	pH of the bacteria suspension	Challenge dose
1	8.5	10	8.5	
2	6.5	10	6.5	
3	5.5	10	5.5	1.06×10^7 cfu ml^{-1} for 30 seconds
4	7.5	10	7.5	
Un-inoculated control	7.5	10	7.5	Saline water 0.85% for 30 seconds

4.3.2.4. Experiment design of the effect of salinity to the *E. ictaluri* infection on *P. hypophthalmus*

Bacterial preparation

The *E. ictaluri* challenge inocula were grown in 50 ml of sterile TSB with 4 different NaCl concentrations of 0%, 0.5%, 1%, and 1.5% and grown in a shaking incubator at 28°C, 140 rpm for 24 hours. The NaCl concentration of TSB for this test was adjusted by adding NaCl (Oxoid, UK).

Experimental design

The experimental design is provided in Table 4.3 with 4 treatment groups. Fish were held at 0.5, 1 or 1.5% NaCl at stocking density of 10 fish per tank for 2 weeks before challenge with bacteria grown in TSB with 0, 0.5, 1, 1.5% NaCl. Each treatment group was conducted in triplicate and an un-inoculated control (0% NaCl) receiving the same treatment but not bacteria was included.

Table 4.3. Experimental design of effects of salinity on *E. ictaluri* infection in *P. hypophthalmus*

Treatment group	% NaCl in water	No.fish/tank	% NaCl in water	Challenge dose
1	1.5	10	1.5	1.1×10^7 cfu ml^{-1} for 30 seconds
2	1.0	10	1.0	
3	0.5	10	0.5	
4	0	10	0	
Un-inoculated control	0	10	0	Saline water 0.85% for 30 seconds

All the experimental challenge studies were performed in the Wet Laboratory of the International University, Ho Chi Minh National University, Vietnam in 50 L plastic tanks using continuous flow-through water at 0.38 L minute^{-1}, a 12 h light: 12 h dark cycle and water temperature at 26 ± 2°C for 15 days. Aeration was supplied through an air stone to each tank and the fish were fed with a commercial diet (Catfish 2 T502, Uni-President Co., Vietnam) to apparent satiation twice daily. The challenge facilities were flow-through so the desired pH and salinity of water were adjusted by addition of HCl 1N or KOH 1N or NaCl (Oxoid, UK) before being provided to each treatment group. The water temperature, salinity, pH and mortality/morbidity was checked and recorded 4 times per day and fish received a maintenance feed ration twice daily until the first

mortality was observed. Bacterial and histology samples from moribund and fresh dead fish were conducted as previously described in chapter 3.

At the end of the challenge period, 50% of all surviving fish per treatment group were removed. These were examined for gross clinical signs of disease and sampled for bacterial recovery as already described in chapter 3.

4.3.3. Statistical analysis

Parametric assumptions (optical density OD and Cumulative mortality percentage) were analysed using ANOVA. The samples with homogenous variances were analysed using the Duncan test, while Dunnett´s T3 test was used for the samples with unequal variances. All tests were performed using SPSS program release 17.5.

4.4. Results

4.4.1. Biophysical characteristics of pH and salinity tolerance of *E. ictaluri.*

All bacterial isolates examined in this study grew in broth at 0 to 2% NaCl and no growth or viable recovery was observed in the bacterial culture medium treatment groups at 2.5 % and 4% NaCl (Figure 4.1).
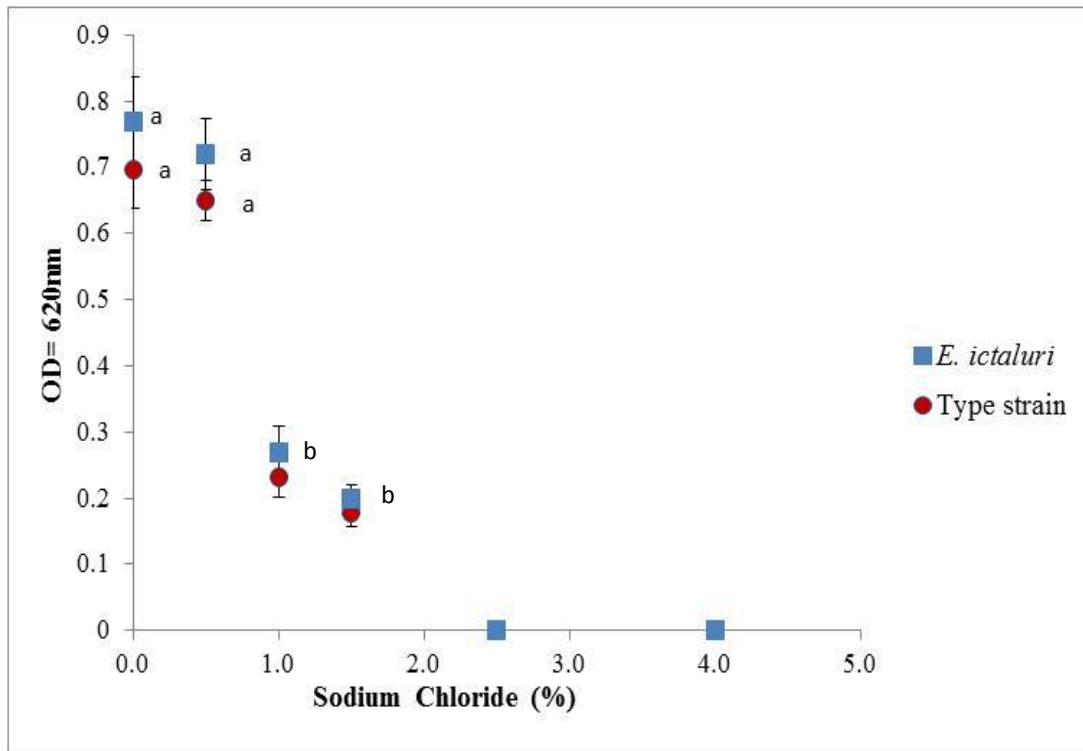
Figure 4.1**.** The viable growth of *Edwardsiella ictaluri* grown in 0-4% NaCl, *in vitro*. Means with the same letters are not significantly different (p<0.05).

Although *E. ictaluri* grew in NaCl concentration of 0 to 1.5%, optimum growth of NaCl

concentration was between 0-0.5% as determined by absorbance values (Fig 4.1) and

viable recovery. Viable and cultured growth was significantly higher for those isolates

at 0 and 0.5% NaCl compared with those at 1% (p=0.02; p=0.01) and 1.5% (p=0.01).

Whilst the organisms remained viable at 2.5% NaCl, they became non-culturable,

which was reversible for the strains at this salt concentration after inoculating onto

normal TSA (0.5% NaCl) but non-reversible for higher salt concentrations tested. All

isolates grew in the culture media with pH from 4.5 to 8.5 but bacterial recovery was

better at pH 5.5 and 6.5 compared with other pHs (Figure 4.2). Growth of the isolates

examined was statistically greater at pH 6.5 than at pH 4.5 (p= 0.001), 5.5 (p=0.01), 7.5

(p=0.008), 8.5 (p=0.001) and 9.5 (p=0.001) (Figure 4.2). Vietnamese isolates had a

better viable growth rate than the USA NCIMB type strain at pH 5.5 (p= 0.000) and 6.5 (p= 0.001). All isolates remained viable but non-culturable at pH 9.5, they became culturable once resuscitated onto normal TSA (0.5% NaCl) after 3 days at 28°C.
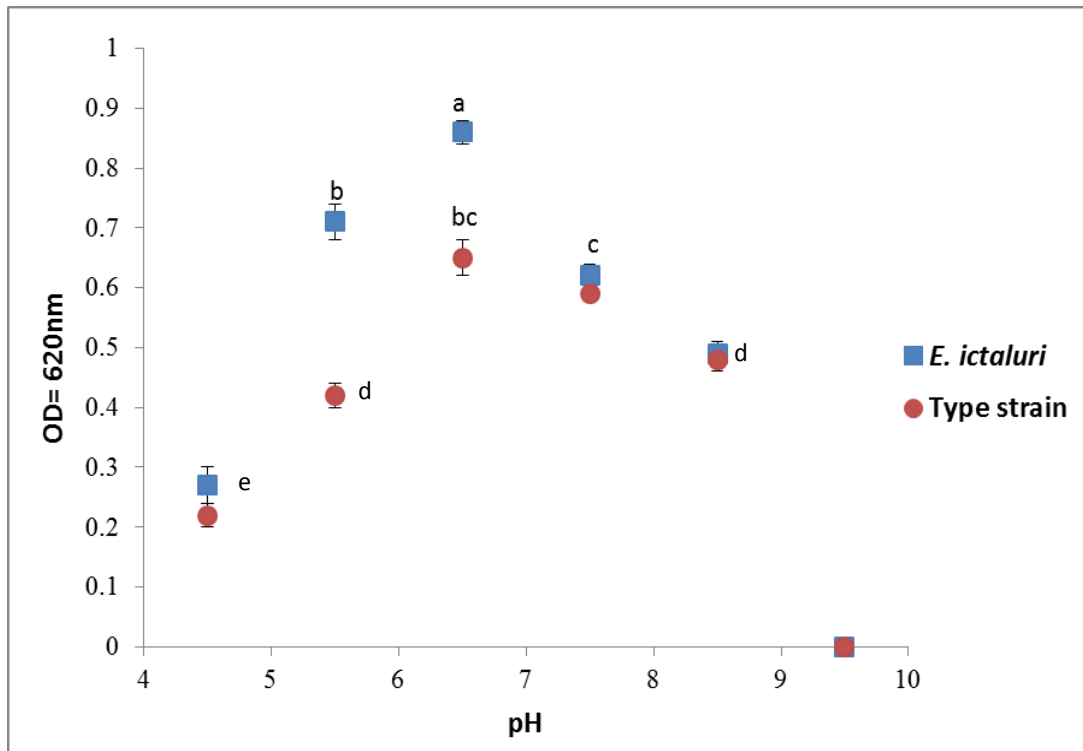
Figure 4.2. The viable growth of *Edwardsiella ictaluri* in different pH conditions*, in vitro.* Means with the same letter are not significantly different (p<0.05).

4.4.2. Effect of the pH and salinity on the infection of *E. ictaluri* in striped catfish.

The results of the challenge experiments showed that both salinity and pH significantly affected the *in vivo* infection of *E. ictaluri* in striped catfish as determined from the cumulative percentage mortality (Figure 4.3 & 4.4, respectively).

The highest mortality (100%) was recorded in the treatment group held in 1.5% NaCl and 90% mortality was observed in the group at 1% NaCl (Figure 4.3). Mortality was significantly greater in the 1.5% and 1% NaCl treatment groups than in 0% NaCl

(p=0.01 and p= 0.02 respectively) and those in 0.5% NaCl (p=0.005 and p= 0.01 respectively) treatment groups (Figure 4.3). Total cumulative mortality was lowest in the 0.5% NaCl treatment group (Figure 4.3).
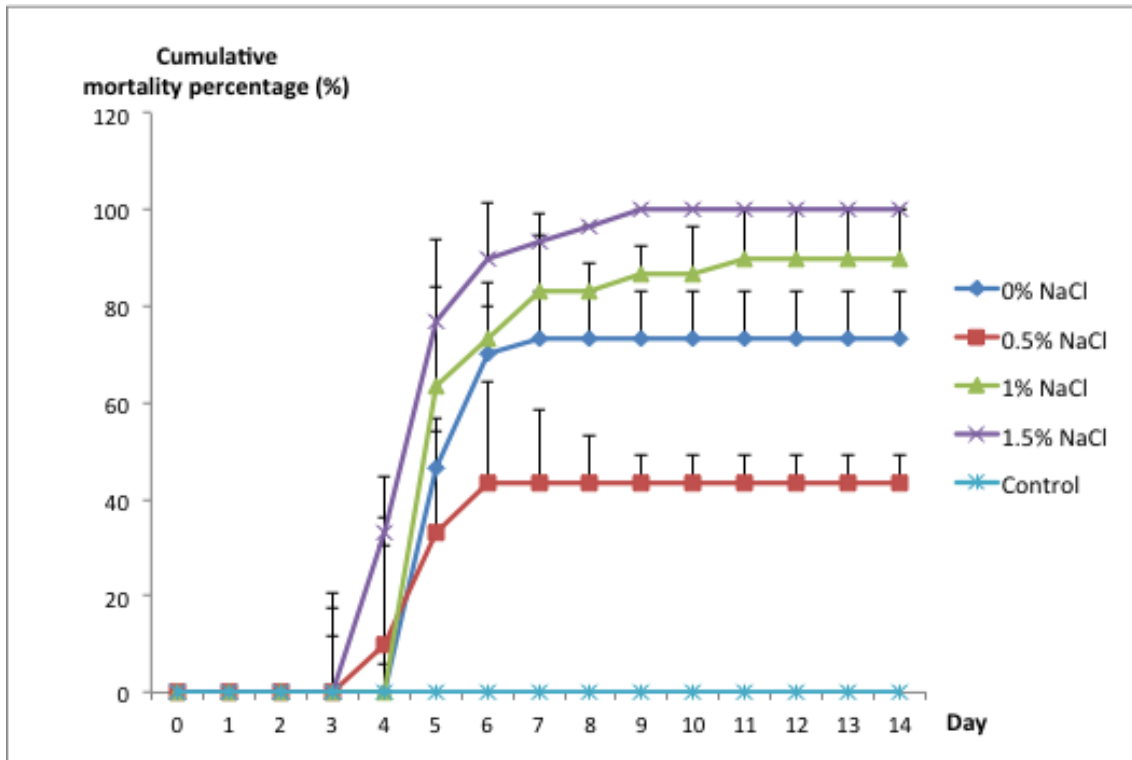
Figure 4.3. Cumulative mortality of striped catfish in different salinities after immersion exposure to *E. ictaluri* for 30 seconds.

Mortalities were observed in all fish groups receiving the bacteria at all pH values used in this study (Figure 4.4). The highest total mortality (98%) was observed in the group exposed to the bacteria at pH 5.5 and was significantly greater than those at pH 6.5 (p=0.02), 7.5 (p=0.02), and 8.5 (p=0.001) (Figure 4.4).

Although the mortality in the treatment group that had been exposed to the bacteria at pH 6.5 was higher than the group exposed at pH 7.5, there was no significant difference between these two groups (p=0.09). In contrast, the cumulative percentage mortality of fish exposed to bacteria at pH 8.5 was lowest (49%) and significantly lower

than other bacterial treatment groups at pH 5.5 (p=0.001), 6.5 (p= 0.02) and 7.5 (p=0.02).
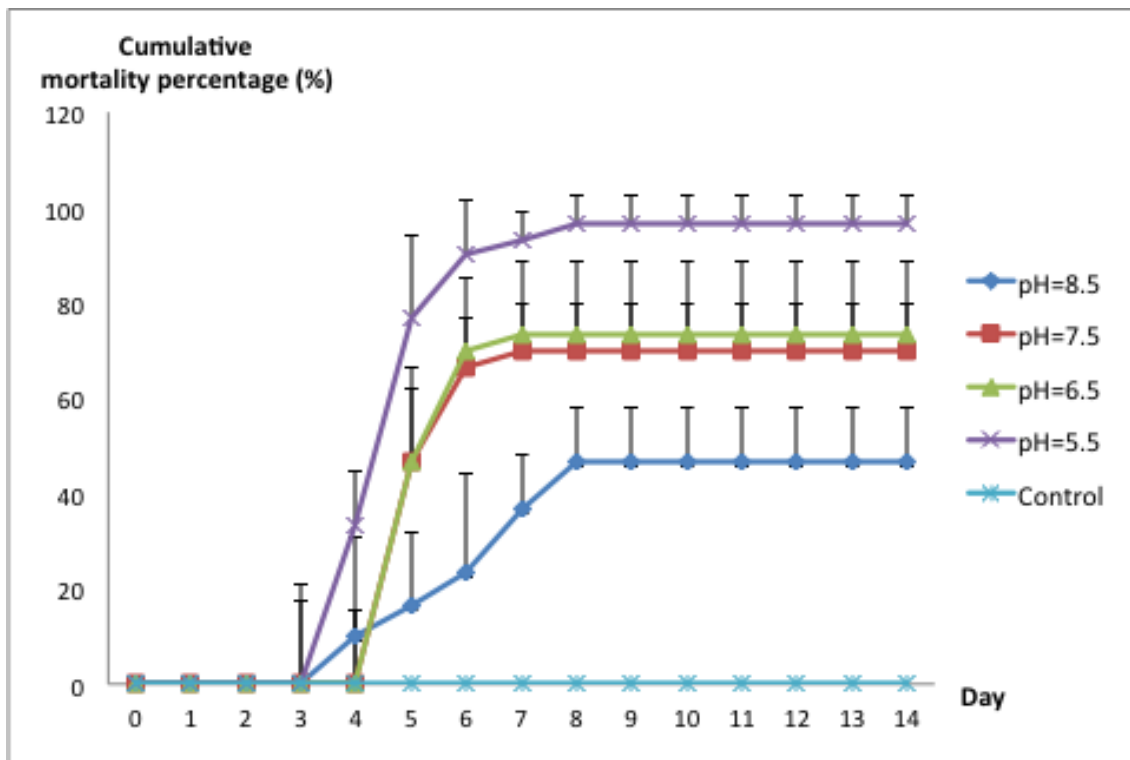
Figure 4.4. Cumulative percentage mortalities in fish exposed to *E .ictaluri* for 30 seconds under different pHs and the control group.

The mortality curves for each of the bacterial treatment groups under different pH and salinity conditions were similar. The first mortality occurred at day 3 post bacterial challenge in the treatment groups pH 5.5 and 6.5 and in the treatment groups receiving 1 and 1.5% NaCl. The mortality continued to increase to day 7 and then stopped at day 10 post challenge. There was no mortality or morbidity in any control group (Figure 4.3 & 4. 4).

4.4.3. Clinical signs and gross pathology.

Fish experimentally infected with *E. ictaluri* under different pH and salinity conditions showed clinical signs of BNP disease similar to naturally infected fish (Crumlish et al. 2002; Crumlish et al. 2010) with typical clinical signs of white lesions observed in the kidney and liver within 4 days post exposure (Figure 4. 5).

No mortalities/morbidity or clinical signs of disease were seen in the control fish group or the survivors.
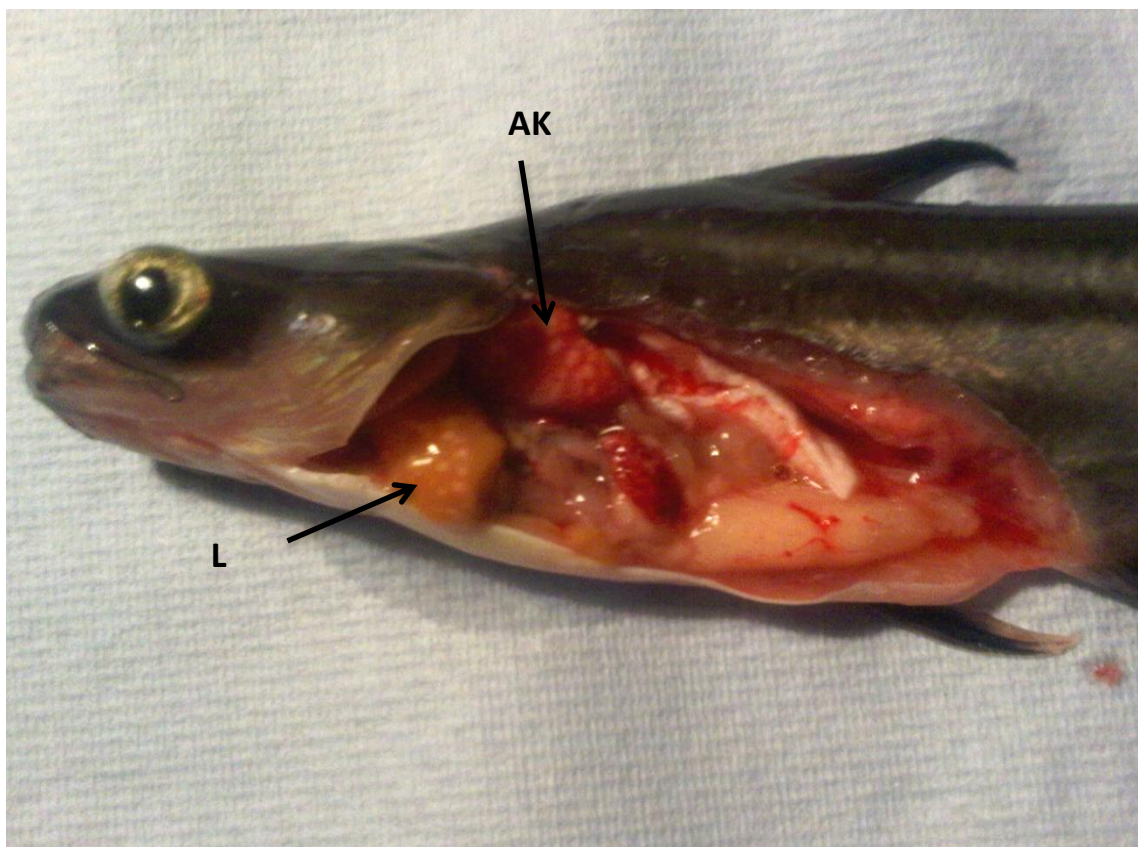
Figure 4. 5. White lesions observed in the anterior kidney (AK) and liver (L) of infected fish.

4.4.4. Bacteria phenotypic identification.

All bacterial isolates recovered from affected fish with clinical signs of BNP from all experiments performed were very homogeneous and showed almost identical

phenotypic characteristics to the original challenge strain. All isolates were described as small Gram-negative rods, formed semi-transparent, round colonies on TSA and were cytochrome-oxidase negative. They were positive for lysine decarboxylase and only fermentation was observed using glucose as the substrate. The API 20E biochemical profile was 400400 for all isolates recovered during the experimental challenge studies performed, which confirmed *E. ictaluri*. Pure cultures were recovered from the kidney of moribund fish in all experiments where fish had clinical signs of BNP disease. Especially in the treatments of pH 5.5 and the treatment of 1% NaCl and 1.5% NaCl pure cultures were obtained in fresh dead fish. Moreover, pure *E. ictaluri* recovery was not only obtained from kidney and liver but also from the brain of infected fish held in 1 and 1.5%. This was not observed in any other salinity or pH treatments. Mixed colonies were observed with un-fresh dead fish but only one sub culture was required for pure bacterial recovery from the dominant colony morphology. No *E. ictaluri* was recovered from the surviving or control fish sampled at the end of the study period for any experimental groups.

4.4.5. Histopathology.

Infected fish exposed to different salinities showed significant pathological changes in the gill and/or skin. The skin of un-infected fish (Fig 4.6A) showed three distinct layers with a superficial squamous epithelium, the majority of the epidermis was mainly composed of club cells and a basal layer containing a lot of lymphocytes. The squamous cells of fish in the treatments at 1 % (Fig 4.7 A) or 1.5 % NaCl (Fig 4.7B) showed hypertrophy compared with control fish (Fig 4.6 A) or fish kept at 0.5% NaCl

(Fig 4.6A). Haemorrhages were observed beneath the epidermis of fish exposed to 1.5% NaCl (Fig 4.7B). The skin of infected fish held at 0.5% NaCl did not show any change in histology (Fig 4.6B). Haemorrhagic lesions were observed in the dermis of affected fish in treatment group pH 6.5 (Fig 4.8A) and these became more pronounced in fish at pH 5.5 (Fig 4.8B). Hypertrophy of the squamous epithelium was also observed in affected fish from treatment groups at pH 6.5 (Fig 4.8A) and pH 5.5 (Fig 4.8B). Fish in treatment groups 1.5% NaCl (Fig 4.9) and pH at 5.5 (Fig 4.10) showed extensive gill hyperplasia. Cells in the epithelial layer of the gill of fish maintained at the high salinity (1.5% NaCl) were seriously affected resulting in rupturing of cells (Fig 4.9). No gill pathology was observed in the control treatment groups. The chromatin in the nucleus of liver cells was distributed irregularly through the cytoplasm indicative of nuclear fragmentation of a cell undergoing apoptosis (Fig 4.11 & 4.12). Cellular inflammation and necrosis were observed in the liver of infected fish in all bacterial treatment groups. Some areas of liver showed the process of karyolysis which resulted in the complete dissolution of the chromatin of a dying cell because of enzymatic degradation resulting in necrosis. This was preceded by karyorrhexis (Fig 4.11B).

Large areas of necrosis and haemorrhage were observed in the spleen and kidney of infected fish from all bacterial treatment groups at all pH and NaCl concentrations (Fig 4.13 & Fig 4.14). Necrotic kidney tubules were observed in all fish exposed to *E. ictaluri* at different pH and salinity conditions (Fig 4.13 & Fig 4.14). Multiple extensive areas of necrosis were observed in the head kidney of affected fish presenting with clinical signs of BNP.

No pathological changes were observed in fish in all control groups.
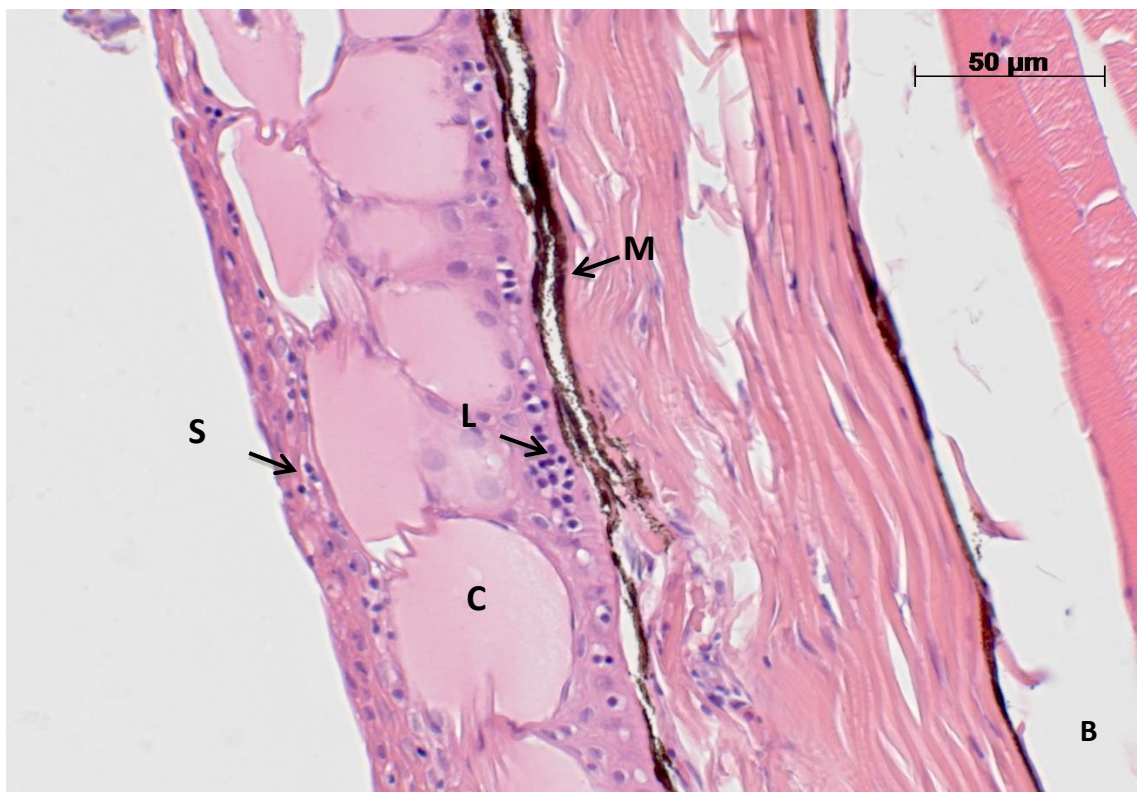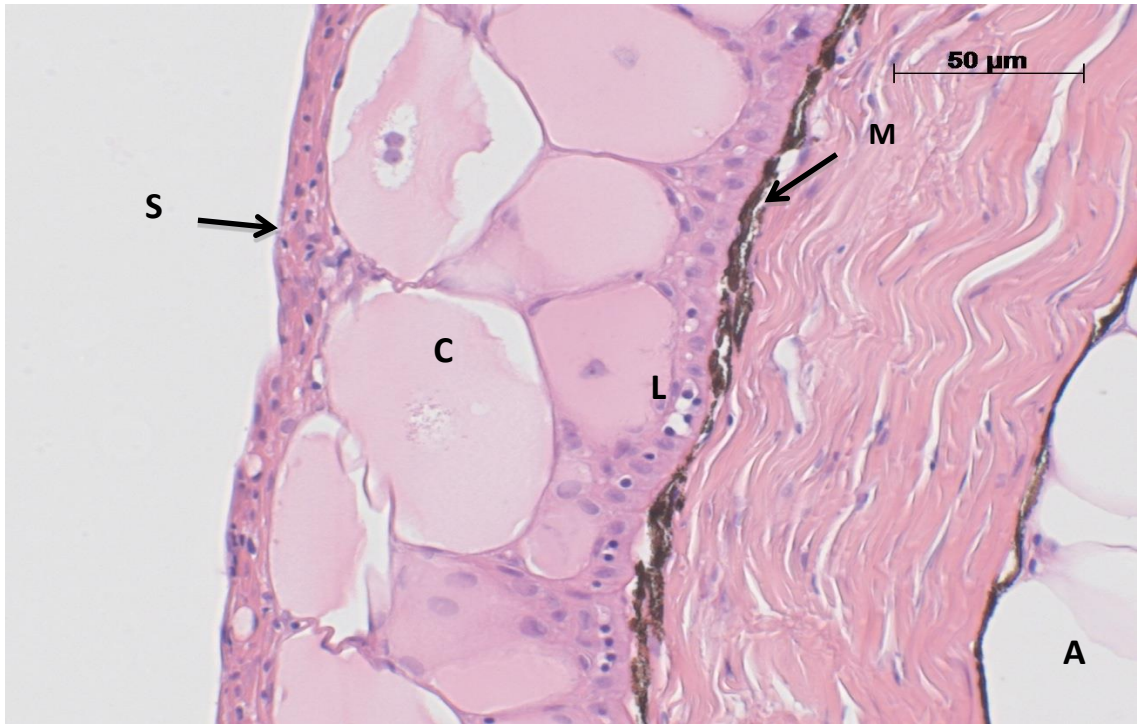
Figure 4.6. Skin structure of un-infected fish held at 0% NaCL in the control group (A) and 0.5% NaCl added (B). The superficial layer is referred to as a squamous epithelium (S). Numerous lymphocytes (L) are in the basal layer. Normal squamous cells in the epithelium layer of the skin of control fish (A) or 0.5% NaCl added (B). Melanin pigment (M) is scattered between the dermal layer and the epidermal layer. C: Club cells.
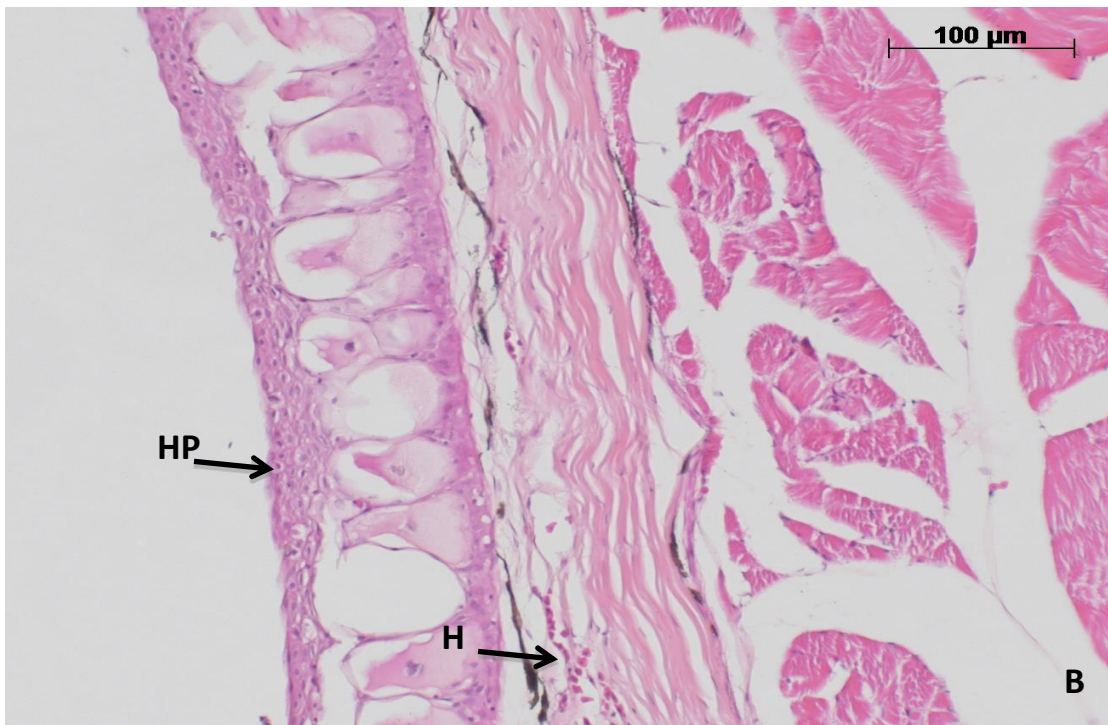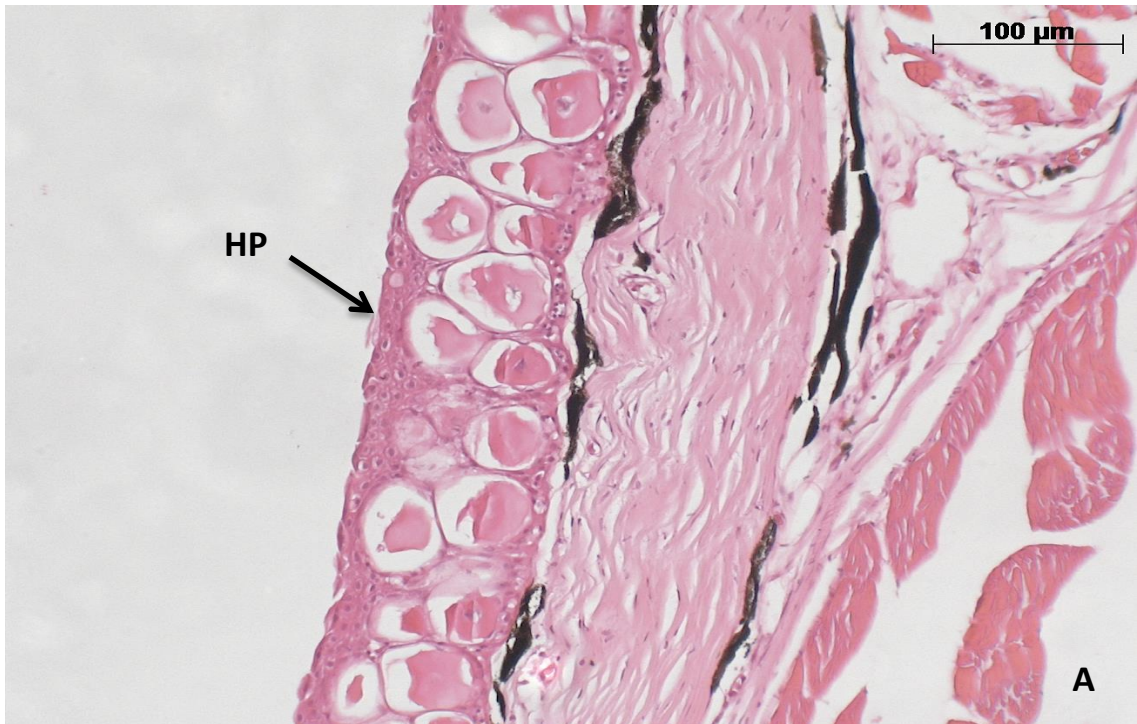
Figure 4.7. A hypertrophic (HP) surface layer was observed in these cells when fish were maintained in higher salinity at 1% (A) and 1.5% NaCl (B). Haemorrhages(H) were seen beneath the epidermal layer at 1.5% NaCl.
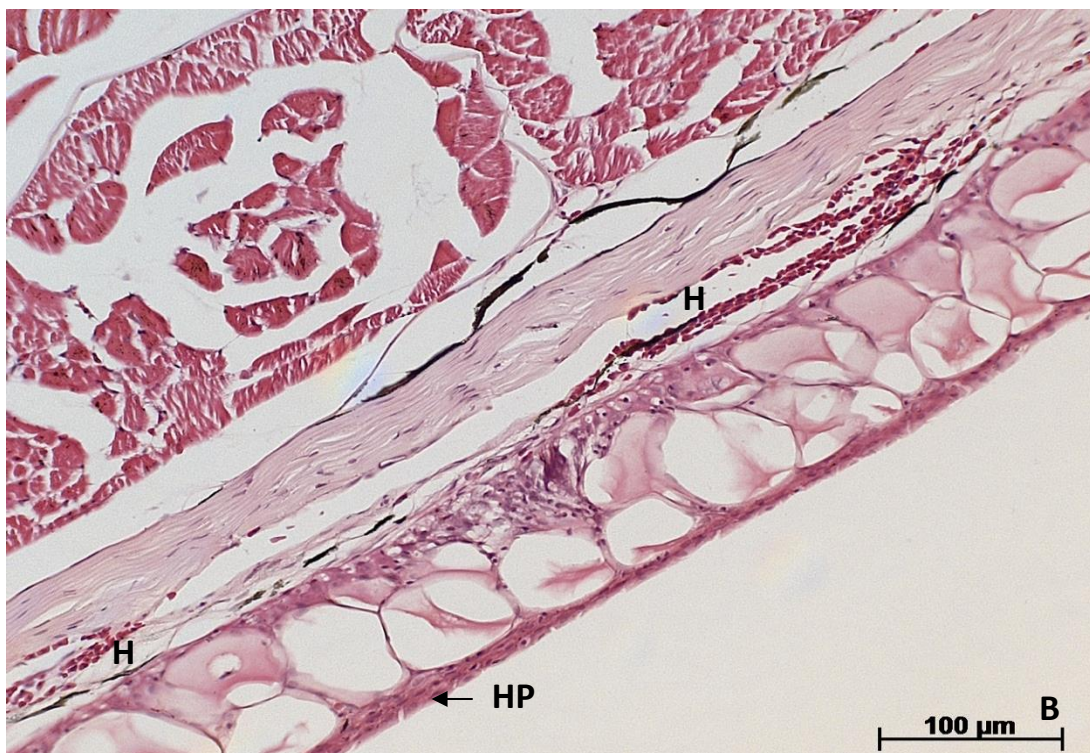
Figure 4. 8. Histopathology changes in skin of infected fish at pH 6.5 (A) and at pH 5.5 (B) showed haemorrhagic areas (H) and hypertrophic cells (HP) in the squamous epithelium layer.
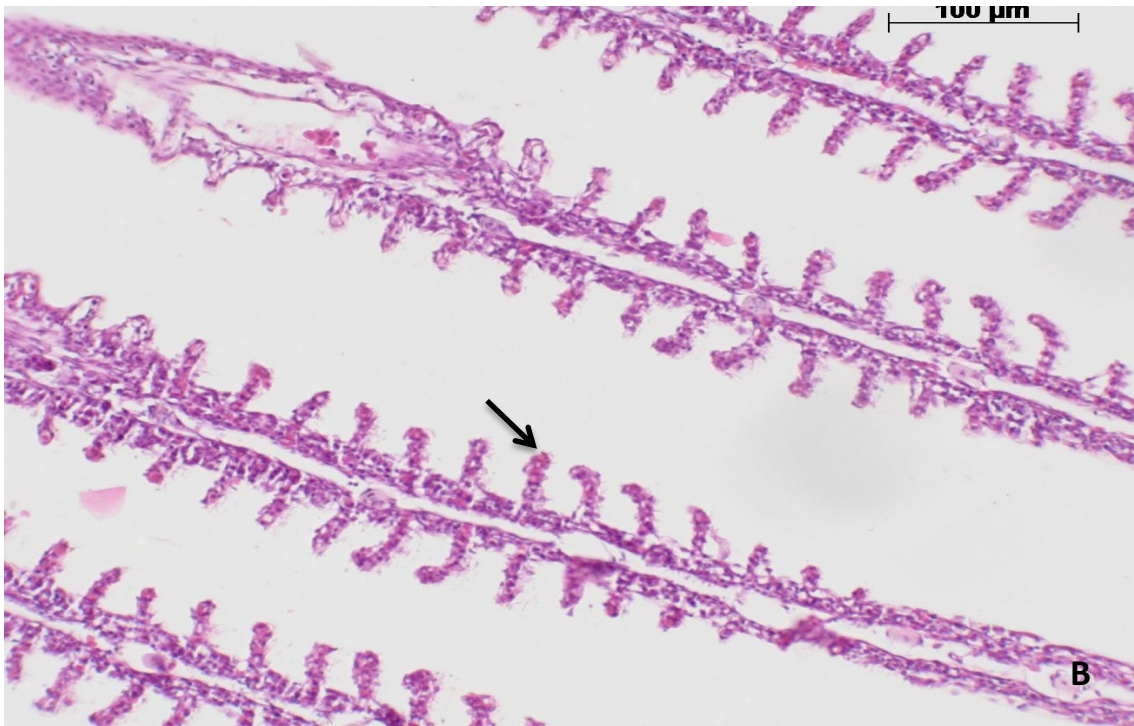
Figure 4. 9. Cytopathology in the gill of infected fish exposed at 1% NaCl (A) and 1.5% NaCl (B) showed hyperplasia (HP, arrow) in the secondary lamellae. Secondary lamellae of fish maintained at 1.5% NaCl showed spongiosis in the epithelium layer (arrow).
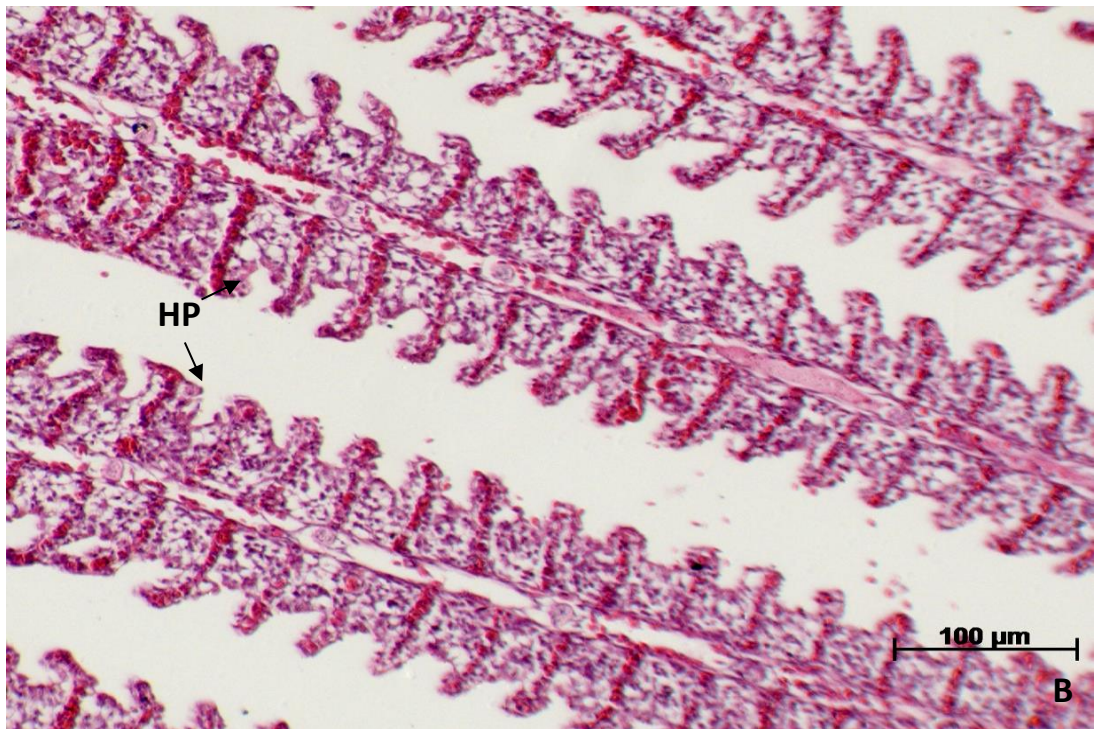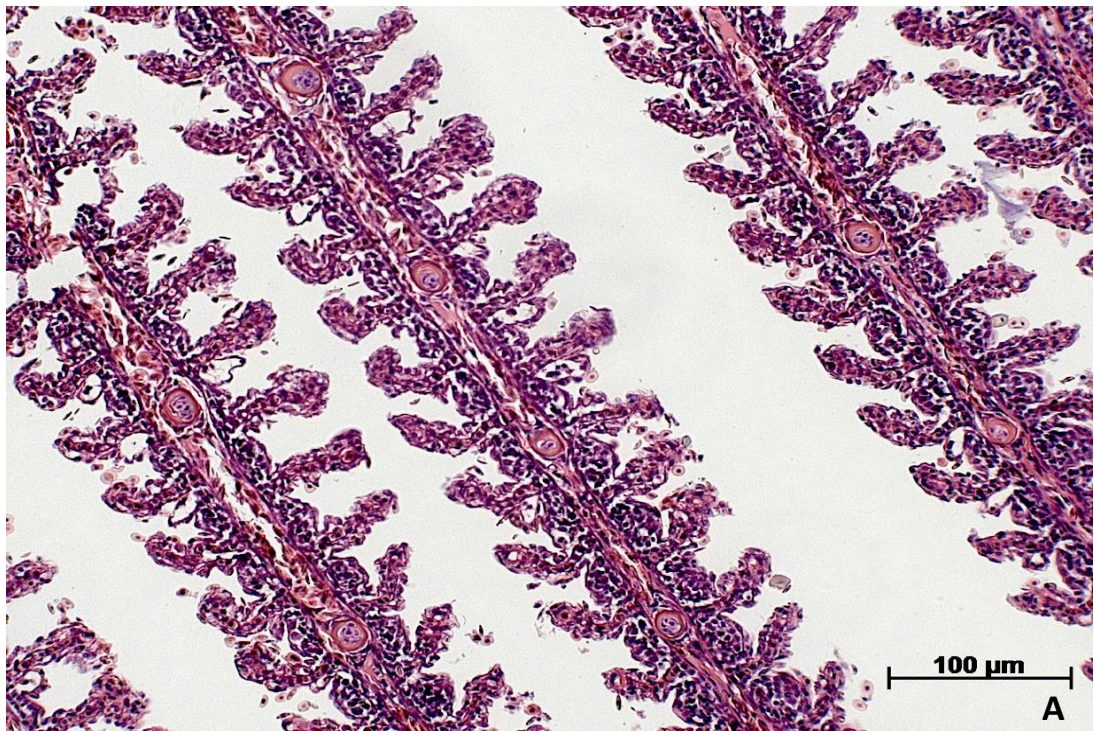
Figure 4. 10. Cytopathology in the gill of infected fish exposed in pH 5.5 (B) showing serious hyperplasia (HP) and spongiosis in the secondary lamella compared with infected fish in pH 8.5 (A).
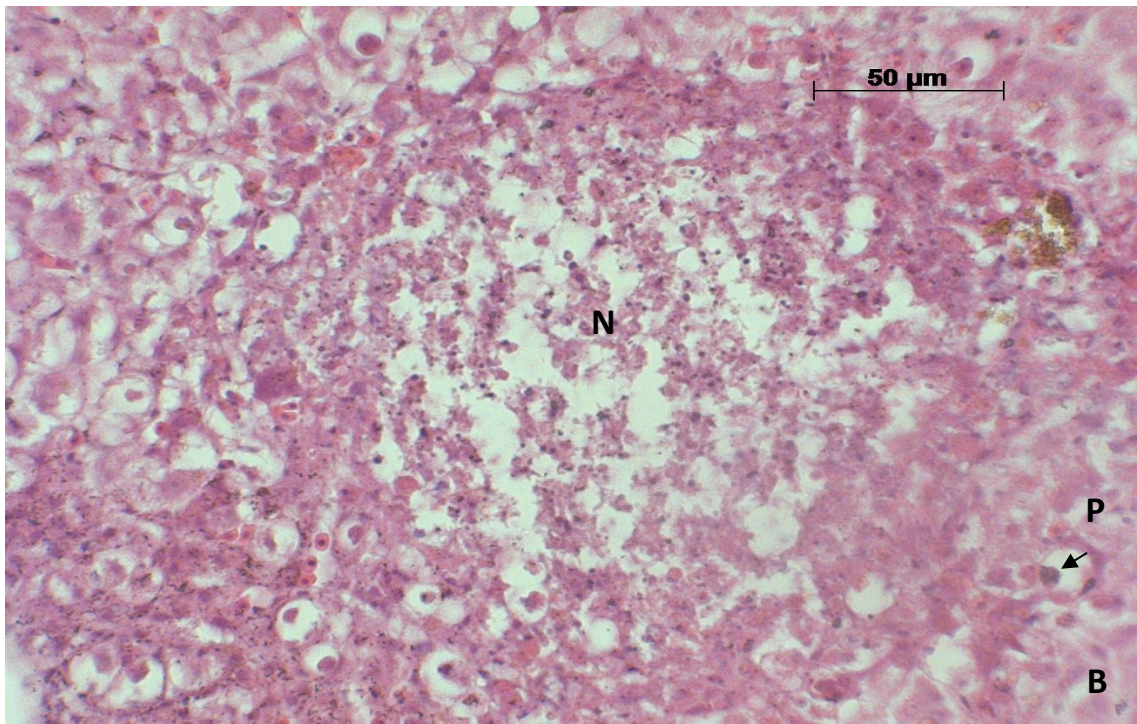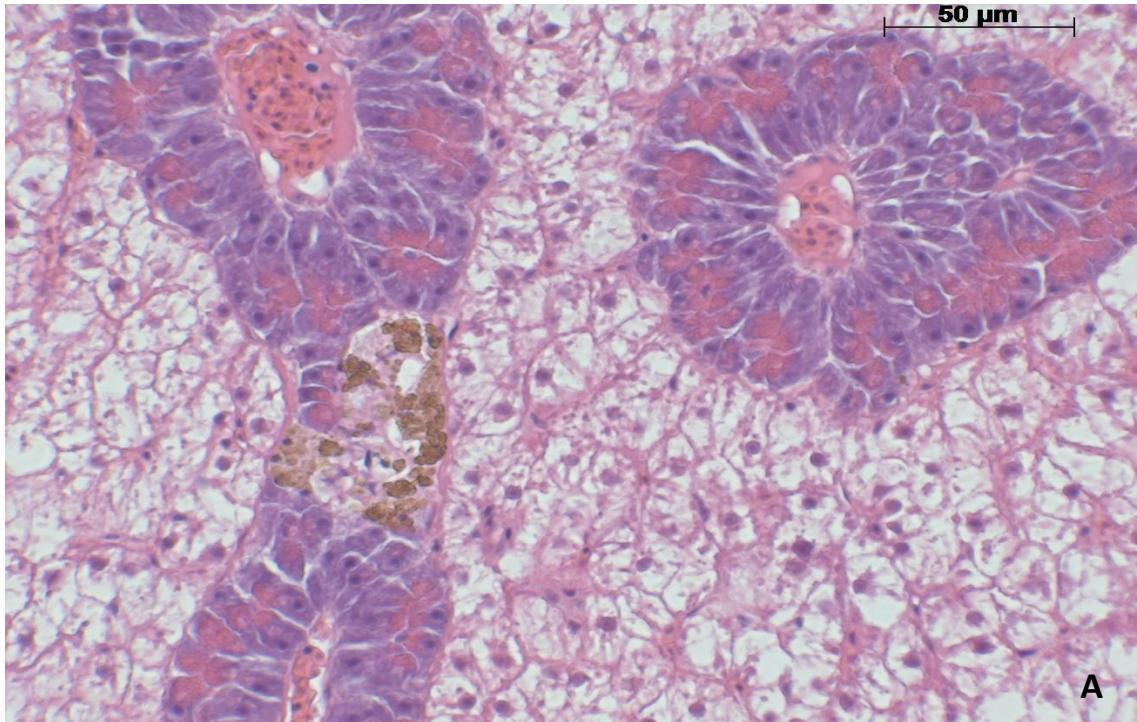
Figure 4. 11. Liver and hepatopancreas cytopathology of fish infected with *E. ictaluri* at 0.5% NaCl (B) showed minor cellular inflammation with some Pyknotic nuclei (P) cells compared with control un-infected fish (A). The liver of infected fish showed severve necrosis (N).
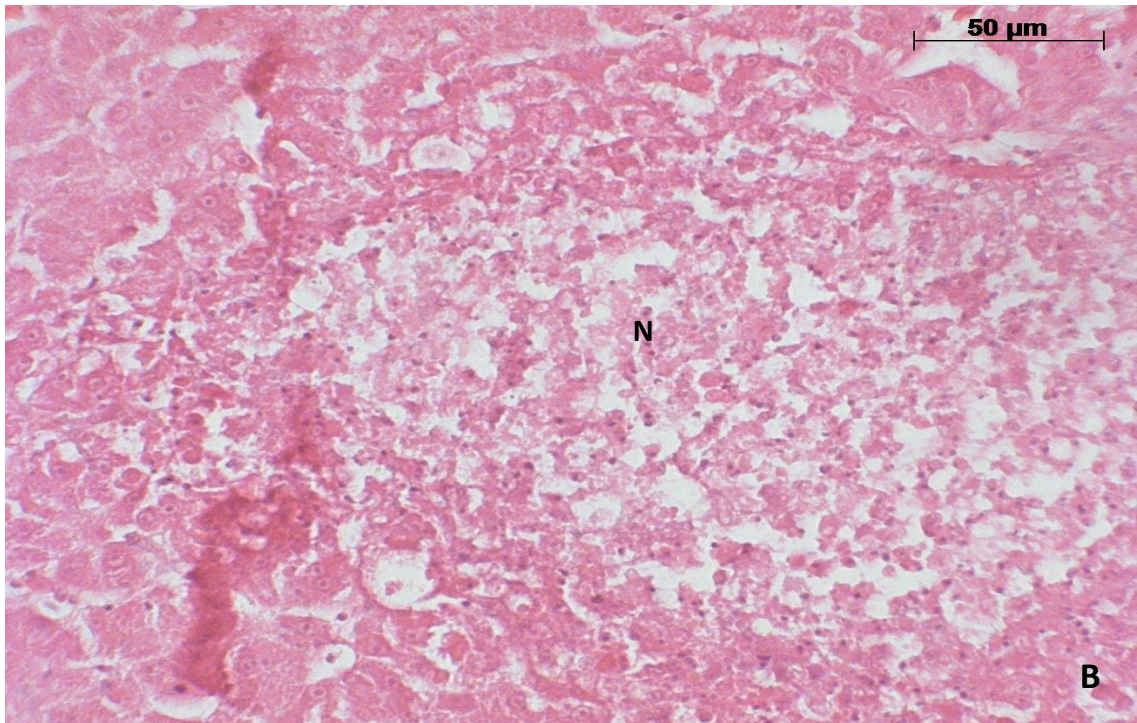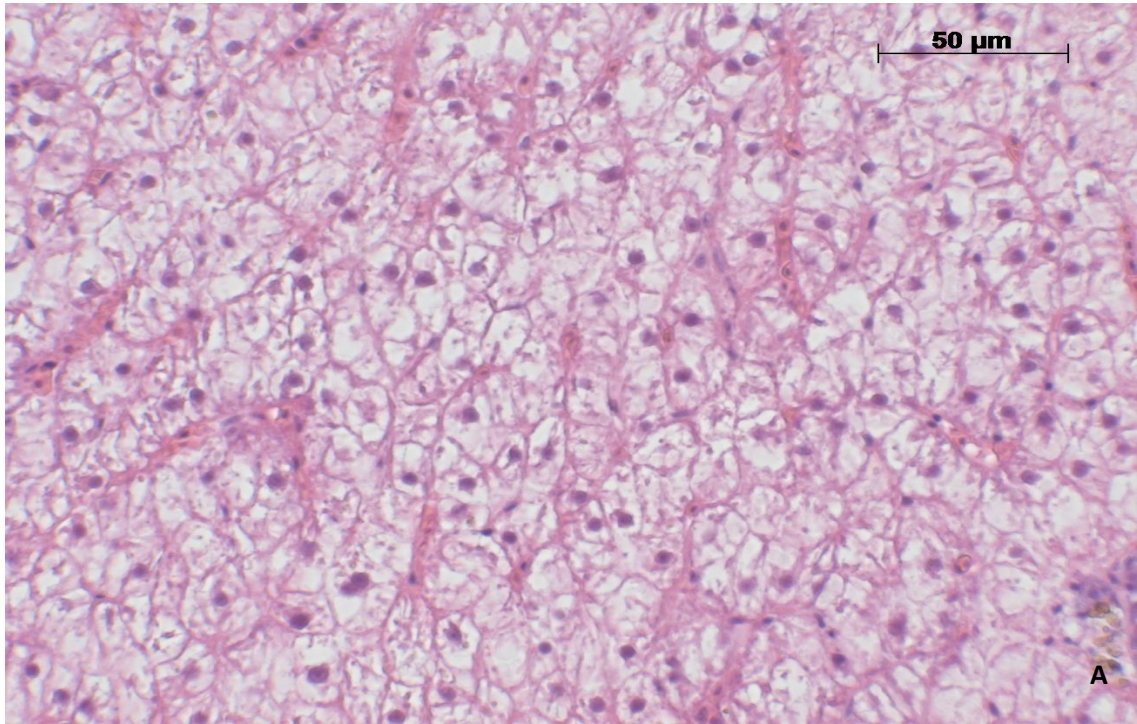
Figure 4. 12. Liver and hepatopancreas cytopathology of fish infected with *E. ictaluri* exposed at pH 5.5 (B) showed necrosis (N) compared with un-infected fish in control groups (A).
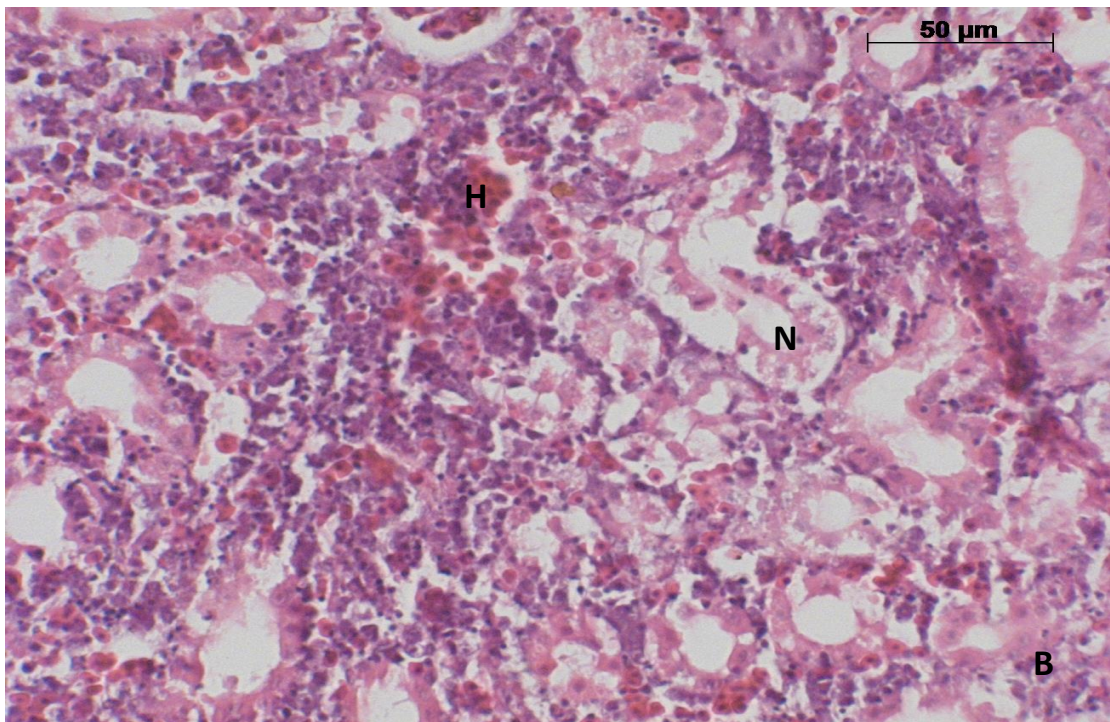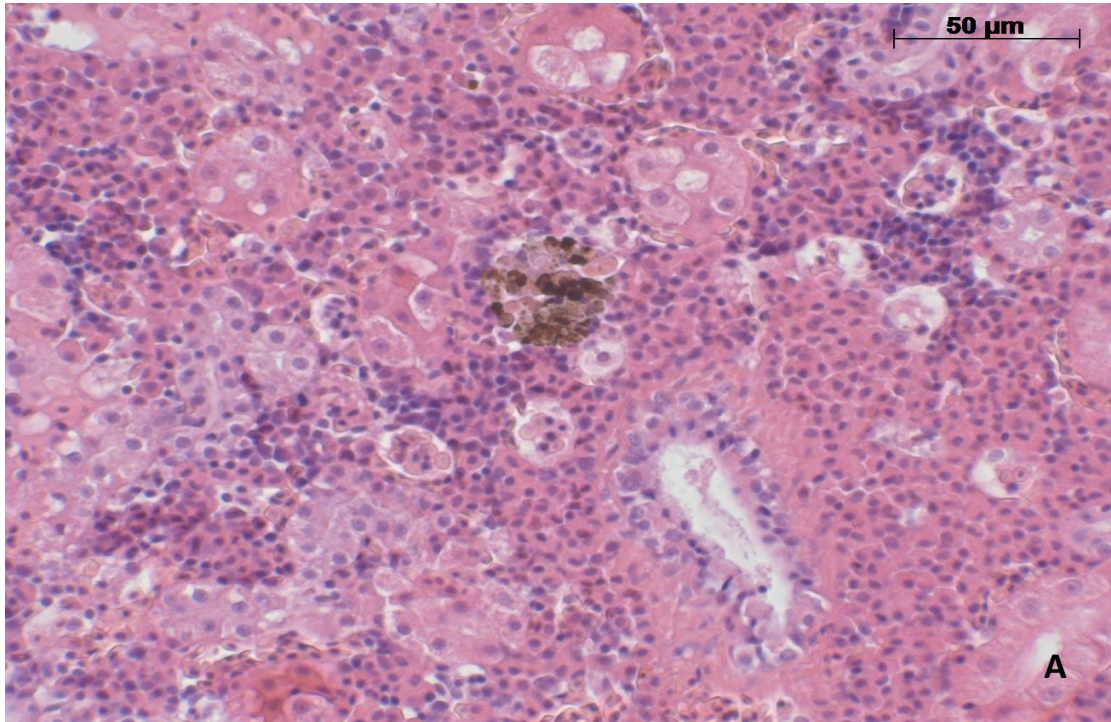
Figure 4.13. Kidney from fish infected with *E. ictaluri* exposed (B) at 1.5% NaCl showed necrosis (N) and haemorrhagic areas (H) compared with un-infected fish in control groups (A).
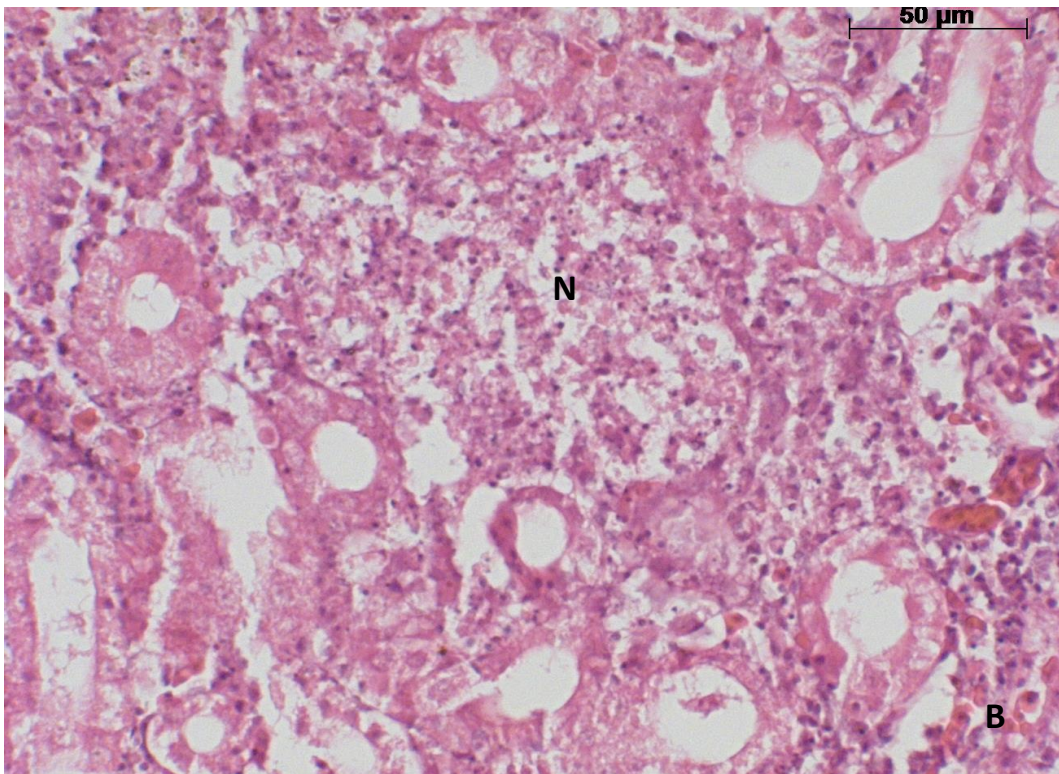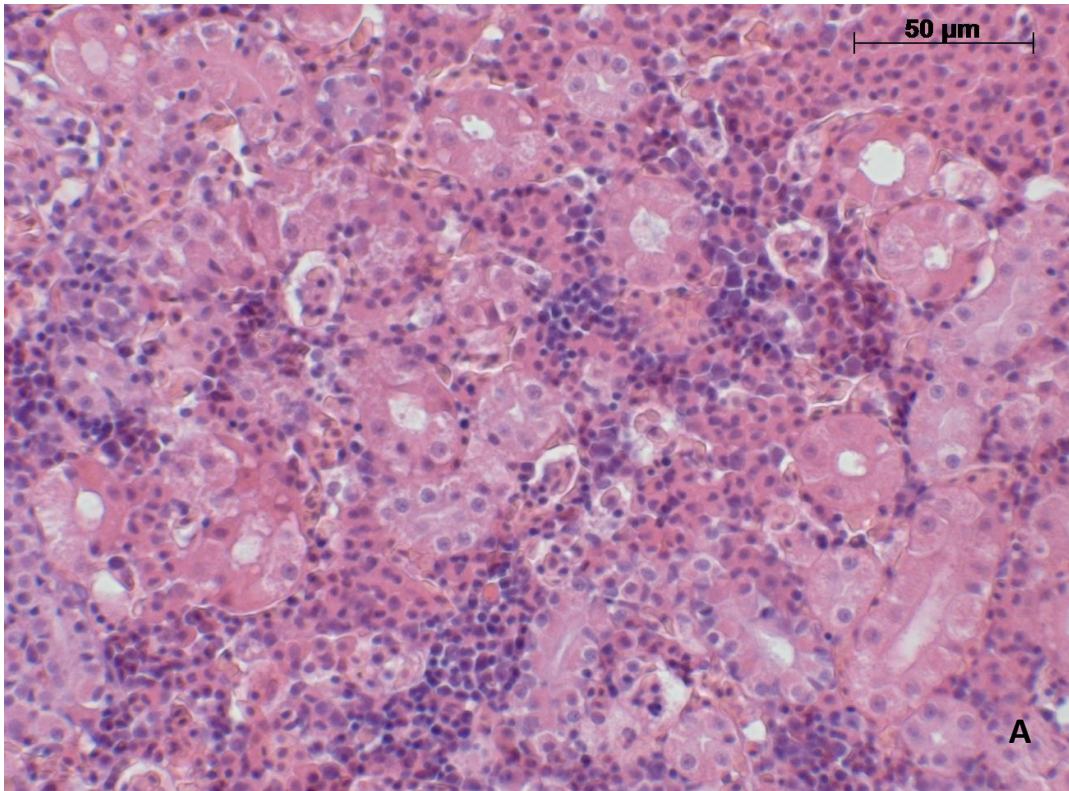
Figure 4.14. Kidney from fish infected with *E. ictaluri* exposed (B) at pH 5.5 showed necrosis (N) compared with un-infected fish in control groups (A).

4.5. Discussion

Striped catfish production in Vietnam has increased in the last decade (Phuong & Oanh 2010; De Silva & Phuong 2011) however, optimal environmental conditions for farming of striped catfish are not well defined and data on the influence of varied environmental factors on disease susceptibility in these fish is scarce. *Edwardsiella ictaluri* is a significant pathogen affecting these fish (Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013) and although production systems have changed dramatically over the last 10 years, disease outbreaks from *E. ictaluri* continue to be a considerable constraint to this sector (De Silva & Phuong 2011; Phan et al. 2011).

In the present study, the results of the tolerance of pH and NaCl concentration from biophysical tests were consistent among the Vietnamese *E. ictaluri* examined and expected given that this is primarily a freshwater pathogen. Sodium chloride tolerance of Vietnamese *E. ictaluri* isolates was similar to that reported from the USA *E. ictaluri* isolates tested (Hawke et al. 1981; Waltman et al. 1986; Plumb & Vinitnantharat 1989). The relatively low NaCl tolerance of Vietnamese *E. ictaluri* isolates investigated in this study was in agreement with the previous findings that *E. ictaluri* can grow in 1.5% NaCl but not in 2% NaCl, thus *E. ictaluri* may be considered more of a freshwater pathogen able to tolerate brackish water conditions (Waltman et al. 1986; Plumb & Vinitnantharat 1989).

Sodium chloride has long been used in the treatment of diseases for freshwater fish (Herwig 1979) and NaCl has been applied in an attempt to improve disease prevention

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during the pond preparation stage and culturing period in striped catfish farming in Vietnam (Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). However, the effect of adding NaCl to striped catfish ponds on the susceptibility of the host and the pathogenicity of the bacterium has not yet been evaluated.

In general, the results of this study showed that the higher the salt concentration added to the water the higher the final mortalities per treatment group. This would suggest a salt concentration depend effect on *E. ictaluri* infection in striped catfish. This is probably due to the host being more susceptible to the salt damage in the gills and skin rather than affecting the pathogenicity of the bacteria *per se*. Significant amounts of gill hyperplasia were observed in the fish at 1 and 1.5% NaCl compared with those kept at 0 or 0.5%. The histology changes observed in gill tissues of these fish would suggest impaired osmoregulation and gas exchange (Ferguson 1989; Ferguson et al. 2001).

Striped catfish is a freshwater fish and the increased water salinity would undoubtedly affect osmoregulation. Gill is an important initial physical barrier considered as part of the non-specific immune mechanism of fish (Madsen & Dalsgaard 1999). Serious hyperplasia and spongiosis in the secondary gill lamellae of striped catfish observed in this study may suggest that fish exposed to high NaCl concentration are under osmoregulatory stress or increased accumulation of toxic metabolites caused by gill damage (Wong et al. 2012). Severe gill damage may lead to interference with the flow of water over the affected lamellar surface resulting in reduction of fish respiration

and increasing disease susceptibility. Thus the increased mortalities observed in the higher salinities are probably exacerbated due to the changes in the host but the point is that the bacteria can survive in these conditions and would be able to infect and spread in fish populations.

In the present study experiments have not been conducted on the invasion of *E. ictaluri* through the gills and their role in pathogenesis, however, higher mortality and pure *E. ictaluri* recovery was only obtained from the brain of infected fish held in 1 and 1.5% NaCl suggesting that at the higher salinities the fish are more severely compromised thus allowing the bacteria to cross the blood-brain barrier. Further work should be done to investigate the connection between the virulence of *E. ictaluri* and high salinity as well as the attachment and invasion of this bacterium to the gills during salinity fluctuations, which might be important in terms of bacterial loading in fish. Further studies should also consider the salinity tolerance of the striped catfish, particularly as reports have shown that salinity of 1.5% NaCl or above can inhibit the growth and survival of striped catfish (PC Thanh pers. comm.). Especially, the fluctuation of temperature and salinity are often more stressful to striped catfish than extremes of these variables (Austin and Austin, 2007; De Silva and Phuong 2011).

Increased NaCl concentration of 0.5% had a positive effect on reducing the mortality of striped catfish infected with *E. ictaluri*. The lowest percentage mortality in the 0.5% NaCl treatment group in this study, was in agreement with the previous findings of Plumb & Shoemaker (1995) who reported that naturally infected channel catfish with

E. ictaluri held in concentrations of 0.1, 0.2 and 0.3% NaCl had a significantly lower mortality (33, 43 and 17%, respectively) than those kept in the 0 or 0.01% NaCl concentrations (100 and 96%, respectively). At present, it is unknown if the mortality of fish infected with *E. ictaluri* held at 0.5% NaCl was low because this concentration had an effect on the pathogenesis or if the striped catfish is more resistant due to improved osmolarity. However, the results of *in vitro* tests showed that *E. ictaluri* was able to grow in the culture media containing 1.5% NaCl suggesting that the striped catfish benefit physiologically from the salt thus increasing bacterial resistance.

Sodium chloride has been commonly used in striped catfish farming as a preventative strategy during disease control (Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). The amount of sodium chloride reportedly used by Vietnamese fish farmers varied from 300kg to 500kg per 20 000m^3 per 1 to 2 weeks (unpublished data). When NaCl is added to the freshwater ponds during the production cycle as a putative preventive measure then this may lead to an increase in the salt tolerance of striped catfish within these aqueous environments. The addition of 0.5% NaCl within the experimental facilities did not significantly affect the behaviour or health of the *P. hypophthalmus* as determined in this experimental study. In Vietnam the use of salt in these farming practices has been considered as a prevention method against *E. ictaluri* infections.

The reduced mortality of fish experimentally challenged with *E. ictaluri* and then maintained in 0.5% NaCl suggested that this level of salinity could be useful for

reducing losses to BNP disease in culture systems where salinity can be easily adjusted. Low salinity can be applied in ponds where water with natural salinity is available. However, the addition of salt to large ponds to obtain 0.5% salinity is costly. Salt bath for preventing BNP disease which would be useful for aquaculture systems where increasing the salinity of culture water is not practical, however, additional research is needed.

Data generated in this study from the *in vitro* work on pH tolerance showed the optimum pH for growth of *E. ictaluri in vitro* was between 5.5 and 6.5 in contrast to the previous finding of Plumb & Vinitnantharat (1989) who found that a pH of 7-7.5 was the optimum growth condition for USA *E. ictaluri*. In this study, Vietnamese isolates grew better at pH 5.5 compared with pH 7.5, as determined by absorbance and viable drop counts. Non–culturable but viable survival of all *E. ictaluri* in the culture medium at pH 9.5 suggested a directly attributable effect of high pH on the cell membrane, ribosomes, proteins and DNA, as demonstrated by Vasseur et al. (1999).

In general, each bacterial species is found to be pH specific i.e. some tolerating low pH while others tolerates high pH (Verma et al. 2011). The viable and culturable growth of *E. ictaluri* isolates in the culture medium at pH 4.5 showed that Vietnamese *E. ictaluri* tolerant with acidic conditions, which is in agreement with Booth et al. (2009). The relationship between pH and virulence of *E. ictaluri* has been investigated where Booth et al. (2009) determined that low pH stimulated the activity of urease required for subsequent proliferation and virulence of *E. ictaluri*. *Edwardsiella ictaluri* is a

urease positive organism and its urease enzyme was only activated by low pH or an acidic environment condition (Booth et al. 2009). Urease plays an important role in the intracellular replication of *E. ictaluri* in head kidney-derived macrophages and possibly through neutralization of the acidic environment of the phagosome that can only be activated at pH 5 (Thune et al. 2007). However, an acidic pH was not required for the initial invasion of *E. ictaluri* in channel catfish (Booth et al. 2009). *Edwardsiella ictaluri* was able to utilize exogenous urea and release ammonia resulting in increasing pH to neutral levels, which enabled replication due to the function of the ammonia and urea transporters in the urease pathogenesis island (PAI) of *E. ictaluri* (Thune et al. 2007). The growth study of Waltman et al. (1986) and Rogge & Thune (2011) showed that although *E. ictaluri* survived at very low pH, it could not replicate at a pH lower than 6 when exogenous urea was not added to the growth medium.

A type III secretion system (T3SS) apparatus gene, T6SS gene and the urease enzyme in both the USA and Vietnamese *E. ictaluri* isolates were required for replication of the bacteria in head kidney macrophages and virulence in catfish was more activated under low pH condition (Thune et al. 2007; Booth et al. 2009; Rogge & Thune 2011; Rogge et al. 2013). Following phagocytosis, the T3SS system trans-locates effector molecules (proteins) directly from the bacterial cytosol to the host cell cytoplasm that is involved in intracellular replication across the phagosomal membrane (Thune et al. 2007; Rogge & Thune 2011). In this study, we did not investigate the expression of urease from bacteria maintained at different pHs nor did we have time to determine the effect of pH on expression of virulence genes. It is important to investigate this

characteristic further particularly as peak *E. ictaluri* infections resulting in heavy mortality in farmed striped catfish in Vietnam have been reported and observed during the rainy season when pH of water was below 6.5 (unpublished data). Furthermore, the availability of a urea source in the fishponds from uneaten feed and fish waste could easily be stimulating the activity of *E. ictaluri* urease resulting in enhanced survival, growth and virulence. Therefore the "BNP window" as described by the Vietnamese fish farmers may be dependent on the pH of the aqueous environment.

In order to illustrate this hypothesis on the pH dependent window of BNP, 4 pHs of water consisting of 5.5, 6.5, 7.5 and 8.5 were used to investigate the effects of pH on the experimental infections of *E. ictaluri* in striped catfish. This pH range was selected on the basis of the report of Wurts & Durborow (1992), who recommended that the pH range for aquaculture should be between 6.5-9.0 and fish may become stressed and die if the pH drops below 5 or rises above 10. Although pH 9.5 can inhibit the growth of this bacterium under *in vitro* conditions it was unrealistic to use this value as it would be dangerous to fish because of the rise in blood NH_3 levels which would result in a marked increase in body stores of total ammonia and toxicity to fish (Randall & Wright 1989).

In the experimental challenge at varied pH values, the striped catfish mortality curve was similar in all groups but the final mortalities were significantly higher at the lowest pH compared with the other treatment groups. Generally, decreased pH leads to

significantly increased mortality of fish during bacterial challenge. Fish exposure at low pH conditions (5.5 and 6.5) also induced gill epithelium damage such as lamellar fusion and oedema as compared with controls at different pH. The initial response to low pH is usually hyperplasia in which the epidermis thickens by producing extra cells (Ferguson 1989; Miron et al. 2008). The thickening of the epithelium leads to the secondary lamellae becoming stuck or clubbed together. Chronic irritation is also likely to cause a thickening of the mucous layer. As a result of this process, fish in all treatments of low pH had problems breathing (Miron et al. 2008) leading to more susceptibility with *E. ictaluri* infection and the progress of disease was more severe compared with those held in water at pH 7.5 and 8.5. Junaidi & Hashida (2010) also concluded that low pH is one of the environmental factors that affect the production of the waste products (e.g. nitrate) in running water systems, which may have a substantial effect on the survival of the catfish. Moreover, freshwater fish can be better adapted to the change of CO_2 and NH_3 gradients across the gills in alkaline conditions by increasing the buffering capacity of the water or reducing CO_2 excretion, decreasing ammonia transfer or excreting nitrogenous wastes as urea (Randall & Wright 1989).

Skin is considered as part of the non-specific immune mechanism of fish and plays an important role in protection against adhesion and invasion of pathogens in fish (Madsen & Dalsgaard 1999). In this study, fish kept at pH 6.5 and at pH 5.5 and exposed to *E. ictaluri* showed large haemorrhagic areas and hypertrophic cells were observed in the squamous epithelium layer of the skin suggesting the destruction of

important initial physical barriers of striped catfish from low pH. Severe skin damage may lead to interference with the non-specific immune mechanism of fish resulting in reduction of protective functions and the adhesion and invasion of *E. ictaluri* through the skin of striped catfish.

The results of this study on the effects of pH on *E. ictaluri* infection in striped catfish agreed with the hypothesis of pH dependence of the "BNP window" in striped catfish.

This is the first study describing the effect of pH and salinity on *E. ictaluri* infection in striped catfish. These data establish a relationship between environmental factors (salinity and pH) and susceptibility of striped catfish to *E. ictaluri* infection. The acidic conditions (pH 5.5 and 6.5) and high salinity (1% or 1,5% NaCl) will result in increased mortality rates of striped catfish infected with *E. ictaluri*. For a tropical country like Vietnam, susceptibility to *E. ictaluri* infection at the low pH and low salinity might be significantly important during the rainy season, hence, good management of water quality may limit the infection of *E. ictaluri* in striped catfish. Further study on the practicality of adding NaCl or lime to striped catfish pond should be done to help to control and prevent BNP.

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Chapter 5. GENERAL DISCUSSION

5.1. Context of this study

Bacillary Necrosis in Pangasius (BNP) disease first described by Ferguson et al. (2001) is still having a severe economic impact on striped catfish farming in Vietnam. Data obtained from natural outbreaks of *E. ictaluri* in farmed striped catfish showed that problems associated with BNP outbreaks often result in high mortality rates particularly in the rainy season, which suggest that environmental factors may exacerbate this condition (Dung et al. 2004; Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). The environment conditions play an important role in all aquatic infectious diseases, however this has not yet been studied for BNP in farmed striped catfish. Perhaps less directly, but nevertheless very important, the fluctuating conditions of environmental factors might have some possible influence on BNP development. Studies performed in channel catfish showed an impact of 0.3% NaCl significantly reducing the mortality of channel catfish experimentally infected with *E. ictaluri* (Plumb & Shoemaker 1995). Moreover, the studies of Thune et al. (2007), and Rogge & Thune (2011) suggested that the virulence and the intracellular replication of *E. ictaluri* in catfish macrophages depended on pH in *in vitro* conditions. In order to understand the complex picture of disease development under different environment conditions, the present study started with a range of bacterial isolates, which were recovered from natural BNP outbreaks in farmed striped catfish of different geographic origins within Vietnam from 2002 to 2011 and were characterized by a wide variety of genotypic and phenotypic parameters. After that, a reliable infection

model to mimic the natural mode of infection was developed to investigate the effects of a variety of environmental factors consisting of pH and salinity on experimental *E. ictaluri* infection in striped catfish *P. hypophthalmus in vivo*.

5.2. Relationsip between *Edwardsiella ictaluri* isolates in Vietnamese striped catfish

It is important to understand the genotypic and phenotypic characteristics amongst pathogenic isolates to detect the emergence of virulent isolates, identify the epidemic spread of microorganisms, and prevent the disease (Panangala et al. 2006b). *Edwardsiella ictaluri*, the causative agent associated with the main disease in cultured ictalurids in the USA, has been considered to be relatively homogeneous (Hawke et al. 1981; Waltman & Shotts 1986; Plumb & Vinitnantharat 1989). BNP due to *E. ictaluri* is a relatively new infectious disease in striped catfish and continues to cause widespread disease through the Mekong Delta in Vietnam (Dung et al. 2004; Crumlish & Dung 2006; Phan et al. 2009; Phan et al. 2011; Luu 2013). It is essential to determine the status of isolates recovered from natural infections over time and geographically distinct farms to get a better understanding about the genetic diversity of *E. ictaluri* strains prevalent within Vietnam, which is important to develop control strategies to prevent or at least reduced the incidence of disease outbreaks. In addition, this could also be used to design programmes of future prevention and control strategies such as the development of a generic vaccine to provide cross-protection against multiple strains of *E. ictaluri* in cultured striped catfish.

In Chapter 2, a range of bacterial isolates recovered from natural disease outbreaks in farmed striped catfish of different geographic origins in Vietnam were conclusively biochemically characterized using a range of assays. In general, *E. ictaluri* isolates from striped catfish in Vietnam have similar phenotypic characteristics to other *E. ictaluri* isolates from infected channel catfish *(Ictalurus punctatus)* in USA (Waltman et al. 1986; Plumb & Vinitnantharat 1989), striped catfish (*Pangasius hypophthalmus)* in Indonesia (Yuasa et al. 2003), rainbow trout (*Oncorhynchus mykiss*) in Turkey (Seçer et al. 2004), ayu (*Plecoglossus altivelis)* in Japan (Sakai et al. 2008), yellow catfish (*Pelteobagrus fulvidraco*) in China (Ye et al. 2009; Liu et al. 2010), Nile tilapia (*Oreochromis niloticus)* (Soto et al. 2012), and zebra fish (*Danio rerio)* in USA (Hawke et al. 2013).

Serology has been applied to identify and investigate the relationship between *E. ictaluri* isolates in USA. An understanding of serotype strain differences would be an important step towards development of vaccines against *E. ictaluri* in catfish (Bertolini et al. 1990). However, it has been widely accepted that *E. ictaluri* strains from ictalurids consisted of a single antigenic group and are serologically homogeneous organisms (Bertolini et al. 1990). Furthermore, the 17 USA *E. ictaluri* isolates from channel catfish and 1 isolate from Tadpole madtom (*Noturus gyrinus* Mitchill) revealed a high degree of homogeneity (70% similarity or higher) in their protein profiles and 95% similarity in their fatty acid methyl esters (FAMEs) (Panangala et al. 2006b). The restricted host range of *E. ictaluri* likely accounts for its adaptive genotype to selectively survive within a favorable eco-niche and hence the uniquely monomorphic

characteristics (Panangala et al. 2006b). It also indicates that, as suggested by Plumb &
Vinitnantharat (1989) identification of distinct strains based on the biophysical,
biochemical and serological reactions was not possible. Therefore, serological
characterization has not been conducted in this study.

Further genotyping of *E. ictaluri* could provide additional information on the
relatedness between the strains. Although there are a variety of methods available,
pulsed field gel electrophoresis (PFGE) has traditionally been considered as the ''gold
standard'' method for bacterial strain typing by some authors (Hyytiä-Trees et al.
2007; Chen et al. 2012). For most bacteria PFGE can resolve DNA fragments with sizes
ranging from about 30 kb to over 1 Mb (Goering 2010). Large restriction fragments are
thus separated in a size-dependent manner and the method yields relatively few
bands on the gel, which makes analysis of the results easier (Sabat et al. 2013). In the
present study, PFGE was used to distinguish putative variations among 80 isolates of *E.
ictaluri* from different outbreaks of BNP in striped catfish. Based on the numerical
analysis of large DNA fragments ranging of 48.5 to 436.5 kb, Vietnamese *E. ictaluri*
isolates could be divided into 6 distinct PFGE groups at high percent (82%) similarity
(Figure 2.3, chapter 2) suggesting that *E. ictaluri* represented a clonal bacterial
population structure by lower levels of genetic and phenotypic diversity (Panangala et
al. 2006b). The results from this study gave 4 more genotype groups compared with
the findings of Bartie et al. (2012) and reflected the strain variation related to province
amongst Dong Thap, An Giang, Can Tho and Vinh Long which are the main catfish
producing provinces in Vietnam. Moreover, the commercial activity of striped catfish

fingerlings, transport of feed and other farm materials amongst the 4 provinces could support the genotype distribution of *E. ictaluri* strains. However, the analysis of PFGE results is prone to some subjectivity and portability of data is limited compared with sequence-based methods (Sabat et al. 2013). Given these limitation it is difficult to assess the evolution of *E. ictaluri* recovered from natural infectious over time, however, distinct changes and genotypes were identifed. Moreover, in Vietnamese *E. ictaulri* particularly, differences in the carriage of a large plasmid (140 kb) have been observed as a single-band difference between the respective PFGE profiles. Therefore, further study of more *E. ictaluri* isolates from different geographical locations, perhaps outwith Vietnam, and from different fish species should be conducted by other molecular typing methods such as Multilocus sequencing Typing (MLST) or a whole genome sequences to provide a better understanding of the epidemiology of this bacterium in striped catfish.

Intensive fish farming in Asia has promoted the occurence of several bacterial diseases, which has led to an increase in the use of antimicrobials (Defoirdt et al. 2011). Vaccination is the best method to prevent bacterial kidney disease (BKD) in salmon and has proved effective in salmonid farming not only as a prevention to infectious diseaes but also contributed towards a reducting in antibiotic use (Sommerset et al. 2005). In the striped catfish sector, vaccination has only recently been commercially available and so antibiotics are still required. Antibiotics have been commonly applied both prophylactically and therapeutically against this disease in the striped catfish farms in Vietnam. A problem of antibiotic treatment used in

aquaculture is the development of drug-resistant bacteria leading to reduced efficiency of antibiotic treatment for human and animal diseases (Frappaolo & Guest 1986). It has been accompanied by an increase of antibiotic resistance in fish pathogens, which undermines the effectiveness of the prophylactic and curative use of antibiotics in aquaculture (Sarter et al. 2007). Antibiotic usage and the emergence of resistant *E. ictaluri* have been demonstrated in farmed striped catfish in Vietnam (Crumlish & Dung 2006; Dung et al. 2008; Dung et al. 2009; Phan et al. 2009; Phan et al. 2011; De Silva & Phuong 2011). Striped catfish farms in Vietnam have spent from 2-5% of the total production cost per season on antibiotics in disease prevention and treatment (Sarter et al. 2007). Defoirdt et al. (2011) previously estimated antibiotic use at approximately 700 g of antibiotic per 500–600 metric tonnes of production in Vietnam. Quinolone, β- lactam, sulfonamide, tetracycline and other combinations were commonly used in striped catfish farming on the basis of the experience of the farmers in Vietnam (Phuong et al. 2005). Multiple antibiotic resistances have been reported in fish pathogens (Schmidt et al. 2001; Teuber M 2001) and particularly in Vietnamese striped catfish (Sarter et al. 2007). In this study, most of the *E. ictaluri* isolates were found to be resistant to oxolinic acid, sulfadimethoxine/ormetoprim (Romet) and oxytetracycline (chapter 2), in agreement with other studies (Suzuki S et al. 2008; Dung et al. 2008; Phan et al. 2011). Oxytetracycline resistance was common in all bacterial species except *Pseudomonas spp.* isolated from aquaculture ponds (McPhearson et al. 1991), especially in oxytetracycline-medicated feed ponds (Depaola 1995) or antibiotic-treated ponds (McPhearson et al. 1991). Similarly, the study of Phuong et al. (2005) showed that among 123 bacterial isolates from water, sediment

and different fish farms (striped catfish, tilapia, common carp and gourami) in five provinces in the Mekong Delta in Vietnam more than 90% of the isolates were resistant to tetracycline, and 89% to trimethoprim-sulphamethoxazole. Bartie et al. (2012) described none of Vietnamese isolates recovered from natural BNP outbreaks from 2001-2005 were resistant to amoxicillin, whilst in the present study more than 60% of isolates from 2007 to 2011 from four provinces were resistant to this antibiotic (Table 2.2; 2.4 and 2.5; chapter 2). This observation suggests that the use of amoxicillin in aquaculture or in aquaculture waters in Vietnam, particularly in these provinces, has led to the development of resistant strains. Importantly, high frequency of multiple antibiotic resistant *E. ictaluri* isolates in Vietnam (Table 2.5, chapter 2) associated with the use of four common antibiotics is similar to that found in the study of Sarter et al. (2007) which described the high recovery of multi-antibiotic resistant Gram-negative isolates from farmed striped catfish reflecting an adaptation of bacteria to the antibiotic pressure in the environment. The emergence of antibiotic resistance among fish pathogens, particularly in *E. ictaluri* isolates (chapter 2) increases the possible transfer of antibiotic resistant isolates to bacteria of terrestrial animals and human, including pathogens (Sørum 2006). The potential impact of antibiotic resistance in striped catfish *E. ictaluri* should be concerned and be further studied.

Plasmids in *E. ictaluri* have been identified and sequenced however this was to determine function rather than to be used as a rapid means of assessing relatedness. The existence of plasmids in different molecular forms such as supercoiled, nicked or linear leads to different migration on electrophoresis and then it is difficult to

determine accurate plasmid size (Rogge et al. 2013). Two plasmids which consist of the 4.8 kbp pEI1 and 5.643 kb pEI2 are consistently present in channel catfish isolates (Speyerer & Boyle 1987; Lobb & Rhoades 1987; Newton et al. 1988; Reid & Boyle 1989; Lobb et al. 1993; Fernandez et al. 2001; Rogge et al. 2013). These two plasmids have been sequenced and published (Newton et al. 1988; Fernandez et al. 2001). Results of this study (Chapter 2) described a 4.0 kb plasmid in *E. ictaluri* in striped catfish. This plasmid was decribed as a hybridisation of pEI1 and pEI2 of *E. ictaluri* in other species of catfish (Reid & Boyle 1989) whereas 4.8 kb (pEI1) and 5.6 kb (pEI2) plasmids have not been found in this study. However, the recent study of Rogge et al. (2013) found both plasmids pEI1 and pEI2 among 19 Vietnamese isolates, therefor the lack of presence in any of the *E. ictaluri* strains investigated during this study should be reconsidered. It might be that the use of restriction enzyme digestion to linearize both nicked and supercoiled forms of the plasmids in the study of Rogge et al. (2013) leads to difficulty comparing their results with results of the current study consisting of both nicked and supercoiled forms. The previous studies described 2 more plasmids, approximately of 35 kb (Bartie et al. 2012) and 140 kb (Dung et al. 2009; Bartie et al. 2012) weight for Vietnamese *E. ictaluri* isolates. Our studies corroborated these findings. Whilst the USA *E. ictaluri* plasmids play an important role in virulence function and do not appear to be involved in conjugative, mobilization or antibiotic resistance functions (Fernandez et al. 2001; Rogge et al. 2013), the role of *E. ictaluri* plasmids in the Vietnamese isolates would appear to be more heavily involved in antibiotic resistance function (Dung et al. 2009; Bartie et al. 2012). The 140 kb plasmid was considered as responsible for combined tetracycline and sulphanomide resistance

determined among Vietnamese *E. ictaluri* isolates (Dung et al. 2009). Therefore, sequencing the Vietnamese *E. ictaluri* plasmids needs to be investigated to detect the antibiotic resistant genes or virulence genes.

Prior to performing the experimental challenge studies, there is a need for reproducible and standardized experimental infection models in order to evaluate the virulence of isolates, to test prophylactic and curative treatments, and to study the host factors influencing the expression of bacterial virulence. Several authors obtained significant mortality (up to 100 per cent) in catfish using *E. ictaluri* following intraperitoneal (i.p.) infection in ictalurid and other fish (Areechon & Plumb 1983; Shotts et al. 1986; Mqolomba & Plumb 1992; Yuasa et al. 2003; Gaunt et al. 2006; Thinh et al. 2009; Ye et al. 2009; Crumlish et al. 2010; Soto et al. 2012; Hawke et al. 2013), or intra-gastric inoculation (Shotts et al. 1986; Baldwin, 1993; Klesius, 1994) or introduction of the bacterium to the nares (Nordmo & Ramstad 1997; Alcorn et al. 2005) or to the gill (Nusbaum & Morrison 1996). The aspects of experimental disease that have been examined intertwined alterations in haematology and serum chemistry values, possible routes of entry of *E. ictaluri* into fish, vaccination testing or gross and necrotic lesions of *E. ictaluri* infection in terminally ill fish. One should realise however, that injecting bacterial cells into the host is not a natural way of infection as it bypasses the intrinsic defence mechanisms of the mucus, skin, gills and gut of the fish (Nordmo & Ramstad 1997; Alcorn et al. 2005). Using the immersion infection model and an inoculation period ranging from 30 minutes to 1 hour, numerous authors likewise enabled the production of the disease in fish (Pasnik et al. 2007; Thinh et al.

2009; Crumlish et al. 2010; Soto et al. 2012). Even then, significant mortalities were only achieved following the induction of severe stress to the fish by exposure to water with stressors such as netting and handling (Ciembor et al. 1995) or inadequate water quality (Wise et al. 1993) or change of temperature (Plumb et al. 1986; Baxa-Antonio et al. 1992; Mqolomba & Plumb 1992), dissolved oxygen concentration (Mqolomba & Plumb 1992) or feed composition (Paripatananont & Lovell 1995). In this study, immersion was the exposure route initially selected, as it is likely to be one of the natural routes of transmission. Exposing fish to bacterial suspensions for short period of time avoids unnecessary stresses for fish (chapter 3). The infectivity in such a model would indicate the capacity of the bacteria to evade the host external defences (Menanteau-Ledouble et al. 2011) and provides a robust challenge protocol that can accurately assess the pathogenesis of this bacterium or the potential treatments. The variation of striped catfish susceptibility to *E. ictaluri* in the various studies including the current study may be due to the bacterial strain, its virulence or genetical characteristics of the fish population.

Following the establishment of an immersion model, a horizontal challenge method (cohabitation) was also developed in this study (chapter 3) to investigate the possibility of providing a more natural alternative challenge method which could then be applied in the subsequent study. Klesius (1994) has been able to produce evidence of horizontal transmission of *E. ictaluri* in channel catfish in a cohabitation experiment. In all experimental cohabitation challenge studies produced in this study (Chapter 3), the *E. ictaluri* isolate chosen was pathogenic to fish and successfully infected striped

catfish when the seed fish were exposed to the bacteria either by direct exposure in i.p. injection or immersion. In this model, mortality was consistently close to 40% within 14 days after cohabitation with the exposed seed fish. Although the end point mortalities were not as high as the suggestion of Amend (1981) the results of this study provided an alternative challenge method for further study in pathogenesis or vaccination of *E. ictaluri.* Cohabitation is considered to mimic natural exposure better than immersion challenge (Garcés et al. 1991; Nordmo & Ramstad 1997) and is considered to be the gold standard in provision of an effective experimental challenge model. In cohabitation challenges, fish do not suddenly interact with unnaturally high numbers of bacteria and then a vaccine that provides a slight immunological advantage to the host should have a measureable effect (Alcorn et al. 2005). One of the crucial elements of this challenge model is infectivity load (Trust 1986). The dose administered to the seed fish by injection or immersion must allow the fish to become infected and not die immediately otherwise subsequent crossover infections could be affected because the fish is already dead (Crumlish pers. com.). To account for the variablity of infectivity load between tanks, cohabitation challenge should be conducted with several replicate test tanks (Amend 1981; Nordmo & Ramstad 1997) suggested that unlike most other animals, less replicates can be used in many fish under favourable environmental conditions, however, at least duplicate test tanks should be used in cohabitation challenges. In this study, cohabitation challenges were designed with duplicate test tanks for all treatment groups that fulfil the requirements of Amend (1981). The results of this study suggested that BNP was horizontally transmitted by *E. ictaluri* carriers (e.g infected striped catfish, dead infected fish).

5.3. Environmental factors and *E. ictaluri* infection in striped catfish

An increase of water temperature, high stocking density, poor environment and management conditions cause physiological imbalances in fish often described as stress, resulting in an increased vulnerability to pathogens (Austin and Austin, 2007; Bromage and Owens, 2009). Although little has been published on salinity or pH tolerances and *E. ictaluri* infections in striped catfish, it appears that environmental conditions (pH, salinity) in the water may favour the expression of virulence factors in certain bacterial pathogens (Rogge & Thune 2011). For example, it has been reported that salinity might play an important role in the adhesion of *Flavobacterium columnare* to the gills of channel catfish *Ictalurus punctatus*, goldfish *Carassius auratus* (Altinok & Grizzle 2001) and Indian catfish *Clarias batrachus* (Verma et al. 2011), enabling them to withstand clearing mechanisms operating on body surfaces (Good et al. 2008). Salinity is also involved in the development of several diseases in cultured fish, such as Edwardsiellosis caused by *Edwardsiella tarda* in Japanese flounder *Paralichthys olivaceus* (Zheng et al. 2004). Channel catfish latently infected with *E. ictaluri* and held in 0.1, 0.2 or 0.3% NaCl had significantly lower mortality than in freshwater (Plumb & Shoemaker 1995). In this study, *E. ictaluri* was able to grow in the culture media containing 1.5% NaCl in *in vitro* tests and an incremental increase in NaCl concentration from 0 to 0.5% significantly decreased the mortality in striped catfish infected with *E. ictaluri in vivo* suggesting that the striped catfish is more resistant due to improved osmolarity.

Water pH was considered as a main factor affecting pathogenicity of some bacteria such as *F. columnare* and *Myxobacterium sp.* in Indian catfish, *Clarias batrachus* (Linn) and *Heteropneustes fossilis* (Bloch) (Verma et al. 2011). Importantly, Thune et al. (2007) found that low pH induced the higher level expression of the gene encoding a putative protein involved in the secretion/activation of a virulence factor in the USA *E. ictaluri*. It is unlikely that *E. ictaluri* is a true obligate pathogen, but its survival is restricted by environmental conditions (Plumb et al. 1986; Plumb & Quinlan 1986; Thune et al. 2007; Rogge & Thune 2011). Moreover, catfish became more susceptible to stress-induced Edwardsiellosis when fish were subjected to rapid environmental condition changes (e.g. temperature, oxygen, salinity, pH…) (Mqolomba & Plumb 1992; Baxa-Antonio et al. 1992; Zheng et al. 2004). In pH experimental challenge studies (Chapter 4), the mortality of *E. ictaluri* infected fish showed a strong correlation with water pH. High mortality was recorded in infected fish reared at pH 5.5 and 6.5 whilst low mortality was noted in a water pH of 8.5. The explosion of striped catfish farming over a decade has resulted in increased water pollution (Anh et al. 2010; De Silva & Phuong 2011). The wastewater and sludge discharge from striped catfish ponds contribute to the acidification of water in the river and surrounding water areas (Anh et al. 2010) and lead to increase in the infection of *E. ictaluri* in striped catfish. Then along with good farming practices, environmental control on pH or salinity might provide a means of controlling BNP disease or at least the bacterial load in the aquaeous farming environment.

5.4. *E. ictaluri* virulence and pathogenesis in striped catfish

In spite of the increased significance of the disease, so far little information exists in the published literature relating to the pathogenesis and the virulence determinants of the Vietnamese *E. ictaluri* isolates. The *E. ictaluri* isolates used in this study were found to be virulent and caused 60-100% mortality in experimental infections. The pathology in gills and skin of infected fish in different pH and salinity concentrations provided primary information on the first steps of the interaction between this pathogen and its host. However, the port of entry of *E. ictaluri* into the striped catfish tissues has not been elucidated yet. Data are especially lacking on the virulence determinant factors in the Vietnamese isolates. The T4SS gene virD4 is an important virulence factor for some bacterial pathogens (Christie & Vogel 2000; Cascales & Christie 2003; Juhas et al. 2008), however, this gene has not been found in Vietnamese *E. ictaluri* (Rogge et al. 2013). The absence of this gene is responsible for this virulence deficiency (Rogge et al. 2013). Thus, little is known about the presence of the full virulence factors and their functionality in the Vietnamese isolates. This gap in knowledge is to a large extent rooted in the difficulties of reproducing the disease under laboratory conditions by contact infection.

5.5. Further work

Edwardsiella ictaluri caused important economic loss in striped catfish farming in Vietnam however the epidemiological implication and manifestations are still unclear. It is also important to know the different pathways of pathogen transmission in order to optimise disease control strategies in striped catfish. These could assess the genetic

diversity or relationship between geography and the host-pathogen interaction involved in *E. ictaluri* infection in striped catfish. The development of a Multilocus Sequence Typing (MLST) scheme for *E ictaluri* would enable the further understanding of the variation of this bacterium because data produced by MLST are unambiguous due to an internationally standardized nomenclature, and highly reproducible. Moreover, the allele sequences and sequence type profiles are available in large central databases that can be queried via the Internet. Furthermore whole genome studies and comparative genomic studies would enable identification of putative virulence determinants.

Knowledge of how the cultural practices enhance the spread of *E. ictaluri* throughout the striped catfish industry is useful. Further field-based studies could identify farm level risk factors, which might be the basis of interventions to reduce the incidence or severity of *E. ictaluri* infection on commercial striped catfish farms.

Additionally, the role and effect of climate, water quality and environmental conditions and other management practices on BNP should be further studied and would aid in better management of this disease. Why *E. ictaluri* is more pathogenic at certain pH and salinity and the nature of primary pathogenesis is another area worthy of study.

5.6. References

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APPENDIX

Appendix 1. Criteria for evaluation of typing methods: definitions.

Criteria for evaluation of typing methods can be divided into two categories: performance and convenience according to Struelens et al. (1996).

Performance criteria

Typeability

The typeability is the proportion of strains that are assigned a type by typing system.

Reproducibility

The reproducibility is the ability of a typing system to assign the same type to a strain tested on independent separate assays.

For complex marker systems, the differentiation criteria used for type assignment should be taken into account. The reproducibility of marker pattern and that of type assignment maybe be different and both need to be evaluated.

To evaluate reproducibility, it is important to design serial experiments that asses the influence of all technical steps involved in type assignment, including: strain preparation (DNA or protein extraction, growth conditions where relevant); test and reagent batch (intratest, intertest); laboratory (inter laboratory, same equipment, standardized protocol); observer interpretation and matching of complex patterns (e.g. DNA restriction or amplification patterns) – inter-observer, computerized assignment versus subjective, visual assignment.

Because reproducibility of a method will greatly affect its discriminatory power, Reproducibility should be >0.95 for all applications, and even higher for reliable definitive typing. Many typing methods offer sufficient within-test reproducibility for comparative typing

of a limited number of strains. However, most molecular typing systems are yet to be shown to be sufficiently reproducible or standardized for use in definitive typing.

Stability

The stability of epidemiologic markers conditions the ability of a typing system to recognize the clonal relatedness of strains derived in vitro or in vivo from a common ancestor strain, despite the phenotypic or genomic variation that may occur during laboratory storage and replication, or during clonal dissemination in nature, especially over prolonged periods or in large-scale epidemics. Because mutations and intra- and intergenomic recombination related to integration or mobilization of plasmid, phage and transposable DNA occur at frequencies depending on species, strain and environmental conditions, the stability of markers testes by every system should be evaluated for every microbial species and ecosystem under study.

The *in vitro* stability is assessed by comparing strains tested before and after longterm storage, and after serial passage on specific culture media.

The *in vivo* stability can be estimated by comparing strains tested before and after passage in a suitable animal model. Such variation must be distinguished from the fortuitous occurrence of different strains in these situations *in vivo*.

Discriminatory power

The discriminatory power is the average probability that the typing system will assign a different type or two unrelated strains randomly sampled in the microbial population of a given taxon.

Epidemiologic concordance

This is the probability that epidemiologically related strains derived from presumably single-clone outbreaks are determined to be similar enough to be classified into the same clones

Typing system concordance

During a single outbreak, multiple clones may be co-transmitted. Therefore, epidemiologic relatedness should be the gold standard for comparing the results of independent typing system clonal delineation and for evaluating typing system specificity. Isolates that are concordantly grouped into highly similar types by several systems are increasingly to be clonally related. In such comparisons, genomics typing systems may in general be given more weight than phenotypic systems. Typing systems exploring polymorphism at multiple sites of the whole genome are more representative that typing systems exploring variation at a single gene locus.

Convenience criteria

Convenience criteria may be important for the selection of appropriate typing system depending on a number of factors, including the scale of the epidemiologic investigation, the timeliness of information needed and the financial and technical resources available. The following criteria may be considered: flexibility, rapidity, accessibility and ease of use. The flexibility reflects the range of species, or higher taxonomic groups, typeable with minimal modifications. The rapidity of typing systems varies from same day to several weeks. Many typing methods can provide results within 24 to 72 hours, which is rapid for most outbreak investigations. Accessibility depends upon the availability and cost of reagents and equipment and the skills required for a given method. The ease of use includes the technical simplicity, the workload, the suitability for processing a large number of strains and the ease of scoring and interpretation of results.

Appendix 2. Summary of isolates origin, year of isolation, PFGE group (A to F), plasmid groups (I to III) and antibiotic resistance to Oxolinic acid (OA), Oxytetracycline (OTC), Amoxicillin (AMO) and Sulfadimethoxine /Ormetoprim (Romet).

Isolate	Origin	Year	PFGE groups	Plasmid group	Antibiotics resistance
001	Dong Thap	2002	B	3	OA
002	Dong Thap	2002	B	-	-
003	Dong Thap	2002	B	-	-
004	Dong Thap	2002	B	-	-
005	An Giang	2002	C	1	OA
006	An Giang	2002	B	-	-
007	An Giang	2002	B	-	-
008	Can Tho	2002	C	1	OA
009	Can Tho	2002	B	-	-
010	Vinh Long	2002	B	-	-
011	Dong Thap	2003	B	-	-
012	Dong Thap	2003	B	3	OA, Romet
013	Dong Thap	2003	B	3	OA, Romet
014	An Giang	2003	B	-	-
015	An Giang	2003	B	-	-
016	Can Tho	2003	B	-	-
017	Vinh Long	2003	B	-	-
018	An Giang	2004	C	2	OA, OTC, AMO
019	An Giang	2004	C	-	-
020	Dong Thap	2004	A	3	OA
021	Can Tho	2004	A	3	OA
022	Dong Thap	2007	D	2	OA, Romet, OTC, AMO

023	Dong Thap	2006	B	2	OA, AMO, Romet
024	An Giang	2002	B	1	AMO
025	An Giang	2004	B	-	-
026	Dong Thap	2005	B	-	-
027	Dong Thap	2005	B	-	-
028	Can Tho	2005	E	1	OA, OTC, AMO
029	Dong Thap	2005	B	3	OA, OTC
030	Dong Thap	2005	B	-	-
031	An Giang	2006	B	-	-
032	An Giang	2006	N.D	-	-
033	Type strain NCIMB 12733				
034	An Giang	2006	B	3	OA, OTC
035	Vinh Long	2005	F		
036	Vinh Long	2005	F	2	OA, OTC, AMO
037	Vinh Long	2005	E	2	OA, Romet, OTC, AMO
038	An Giang	2005	B	1	OA, OTC
039	Dong Thap	2005	C	-	-
040	Vinh Long	2006	F	1	OA, Romet, OTC, AMO
041	An Giang	2006	C	-	-
042	An Giang	2006	B	3	OA, OTC
043	Dong Thap	2006	B	-	-
044	Dong Thap	2006	A	3	OA, OTC
045	Dong Thap	2007	B	2	OA, OTC
046	Dong Thap	2004	A	1	OA
047	Dong Thap	2007	A	-	-
048	An Giang	2007	C	-	-
049	An Giang	2008	C	2	OA, OTC, AMO
050	An Giang	2008	B	-	-

051	Can Tho	2008	C	-	-
052	Can Tho	2009	N.D	-	-
053	An Giang	2009	N.D	-	-
054	Dong Thap	2009	D	2	OA, Romet, OTC, AMO
055	Dong Thap	2009	A	3	OA, Romet
056	Can Tho	2009	D	-	-
057	Dong Thap	2007	D	-	-
058	Can Tho	2008	A	-	-
059	Dong Thap	2008	D	1	OA, Romet, OTC, AMO
060	Dong Thap	2008	D	1	OA, Romet, OTC, AMO
061	Can Tho	2005	C	-	-
062	Can Tho	2010	D	1	OA, Romet, OTC, AMO
063	An Giang	2010	A	3	OA, AMO, OTC
064	An Giang	2010	A	3	OTC
065	Dong Thap	2010	D	-	-
066	An Giang	2010	A	3	OA, AMO, OTC
067	Can Tho	2010	A	-	-
068	Dong Thap	2010	D	-	-
069	Can Tho	2010	A	-	-
070	An Giang	2011	A	3	OA, AMO
071	An Giang	2011	A	2	OA, OT, Romet
072	An Giang	2011	A	-	-
073	Vinh Long	2011	C	1	OA, AMO
074	Vinh Long	2011	C	2	OA, AMO, Romet
075	Dong Thap	2011	D	-	-
076	Dong Thap	2011	E	1	OA, Romet, OTC, AMO
077	Can Tho	2011	C	-	-
078	Vinh Long	2011	C	-	-

079	Vinh Long	2011	A	3	OA, OT
080	Dong Thap	2011	A	-	-
081	Dong Thap	2011	C	-	-

N.D: Not defined; (-) do not study

Appendix 3. Summary of statistical analysis of challenge experiments

1. Establish the challenge model

Descriptives

mortalityday14

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Immersion 30 second	3	63.3333	5.77350	3.33333	48.9912	77.6755	60.00	70.00
Immersion 1 min	3	80.0000	10.00000	5.77350	55.1586	104.8414	70.00	90.00
Immersion 2 min	3	96.6667	5.77350	3.33333	82.3245	111.0088	90.00	100.00
Total	9	80.0000	15.81139	5.27046	67.8463	92.1537	60.00	100.00

Test of Homogeneity of Variances

mortalityday14

Levene Statistic	df1	df2	Sig.
.364	2	6	.709

ANOVA

mortalityday14

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1666.667	2	833.333	15.000	.005
Within Groups	333.333	6	55.556		
Total	2000.000	8			

Post hoc Test

Multiple Comparisons

Dependent Variable:mortalityday14

	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
LSD	Immersion 30 second	Immersion 1 min	-16.66667*	6.08581	.034	-31.5581	-1.7752
		Immersion 2 min	-33.33333*	6.08581	.002	-48.2248	-18.4419
	Immersion 1 min	Immersion 30 second	16.66667*	6.08581	.034	1.7752	31.5581
		Immersion 2 min	-16.66667*	6.08581	.034	-31.5581	-1.7752
	Immersion 2 min	Immersion 30 second	33.33333*	6.08581	.002	18.4419	48.2248
		Immersion 1 min	16.66667*	6.08581	.034	1.7752	31.5581

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

mortalityday14

	Treatment	N	Subset for alpha = 0.05 — 1	Subset for alpha = 0.05 — 2	Subset for alpha = 0.05 — 3
Duncan[a]	Immersion 30 second	3	63.3333		
	Immersion 1 min	3		80.0000	
	Immersion 2 min	3			96.6667
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

2. Environmental factors affecting to the infectivity of *E. ictaluri* in striped catfish.

2.1 pH

Descriptives

Mortality

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
pH 5.5	3	96.6667	5.77350	3.33333	82.3245	111.0088	90.00	100.00
pH 6.5	3	73.3333	15.27525	8.81917	35.3875	111.2792	60.00	90.00
pH 7.5	3	70.0000	10.00000	5.77350	45.1586	94.8414	60.00	80.00
pH 8.5	3	46.6667	11.54701	6.66667	17.9823	75.3510	40.00	60.00
Total	12	71.6667	20.81666	6.00925	58.4404	84.8929	40.00	100.00

Test of Homogeneity of Variances

Mortality

Levene Statistic	df1	df2	Sig.
2.872	3	8	.104

ANOVA

Mortality

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5533.333	3	1844.444	15.810	.001
Within Groups	933.333	8	116.667		
Total	6466.667	11			

Post Hoc test

Multiple Comparisons

Dependent Variable:Mortality

	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
LSD	pH 5.5	pH 6.5	23.33333*	9.12871	.034	2.2825	44.3842
		pH 7.5	26.66667*	9.12871	.019	5.6158	47.7175
		pH 8.5	50.00000*	9.12871	.001	28.9492	71.0508
	pH 6.5	pH 5.5	-23.33333*	9.12871	.034	-44.3842	-2.2825
		pH 7.5	3.33333	9.12871	.724	-17.7175	24.3842
		pH 8.5	26.66667*	9.12871	.019	5.6158	47.7175
	pH 7.5	pH 5.5	-26.66667*	9.12871	.019	-47.7175	-5.6158
		pH 6.5	-3.33333	9.12871	.724	-24.3842	17.7175
		pH 8.5	23.33333*	9.12871	.034	2.2825	44.3842
	pH 8.5	pH 5.5	-50.00000*	9.12871	.001	-71.0508	-28.9492
		pH 6.5	-26.66667*	9.12871	.019	-47.7175	-5.6158
		pH 7.5	-23.33333*	9.12871	.034	-44.3842	-2.2825

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Mortality

	pH	N	Subset for alpha = 0.05 1	Subset for alpha = 0.05 2	Subset for alpha = 0.05 3
Duncan[a]	pH 8.5	3	46.6667		
	pH 7.5	3		70.0000	
	pH 6.5	3		73.3333	
	pH 5.5	3			96.6667
	Sig.		1.000	.724	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

2.2. Salinity

Descriptives

Mortality

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
salinity 0%	3	73.3333	15.27525	8.81917	35.3875	111.2792	60.00	90.00
0.5%	3	43.3333	11.54701	6.66667	14.6490	72.0177	30.00	50.00
1%	3	90.0000	10.00000	5.77350	65.1586	114.8414	80.00	100.00
1.5%	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
Total	12	76.6667	24.24621	6.99928	61.2614	92.0720	30.00	100.00

Test of Homogeneity of Variances

Mortality

Levene Statistic	df1	df2	Sig.
2.872	3	8	.104

ANOVA

Mortality

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5533.333	3	1844.444	15.810	.001
Within Groups	933.333	8	116.667		
Total	6466.667	11			

Post Hoc test

Multiple Comparisons

Dependent Variable:Mortality

	(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
LSD	salinity 0%	0.5%	30.00000*	8.81917	.009	9.6630	50.3370
		1%	-16.66667	8.81917	.095	-37.0037	3.6704
		1.5%	-26.66667*	8.81917	.016	-47.0037	-6.3296
	0.5%	salinity 0%	-30.00000*	8.81917	.009	-50.3370	-9.6630
		1%	-46.66667*	8.81917	.001	-67.0037	-26.3296
		1.5%	-56.66667*	8.81917	.000	-77.0037	-36.3296
	1%	salinity 0%	16.66667	8.81917	.095	-3.6704	37.0037
		0.5%	46.66667*	8.81917	.001	26.3296	67.0037
		1.5%	-10.00000	8.81917	.290	-30.3370	10.3370
	1.5%	salinity 0%	26.66667*	8.81917	.016	6.3296	47.0037
		0.5%	56.66667*	8.81917	.000	36.3296	77.0037
		1%	10.00000	8.81917	.290	-10.3370	30.3370

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Mortality

	salinity	N	Subset for alpha = 0.05 1	Subset for alpha = 0.05 2	Subset for alpha = 0.05 3
Duncan[a]	0.5%	3	43.3333		
	salinity 0%	3		73.3333	
	1%	3		90.0000	90.0000
	1.5%	3			100.0000
	Sig.		1.000	.095	.290

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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